MOLECULAR AND EXPERIMENTAL APPROACHES FOR EXPLORING THE ROLE OF THE SOIL AND ROOT MICROBIOME IN AGROECOSYSTEM FUNCTIONING

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Summary

The world's soils harbor an enormous diversity of microbes, which include communities of bacteria and fungi. This microbial diversity congregates in "hot spots of activity" in the area in, on, and around the roots of plants. These communities of soil and root-associated microbes and their genes collectively function as a microbiome. Because microbes have been shown to play important roles in a number of plant and general ecosystem functions, such as direct plant growth promotion, protection from pathogens, or enhanced nutrient uptake, harnessing the power of the microbiome has been suggested as a way to increase the sustainability of agricultural production. This is particularly important given recent evidence that modern, intensive agriculture is contributing to a variety of environmental issues such as increased nutrient leaching and greenhouse gas emissions, as well as detrimental effects on biodiversity. Managing the soil and root microbiome for the presence of beneficial and/or absence of detrimental members requires knowing how microbial communities respond to agricultural management and elucidating their function both individually and in a community context. However, few studies have focused on examining how both agricultural management practices and different tillage intensities affect both the soil and root microbiome together. Moreover, the functions of many microbes are still unknown. One proposed method for investigating the functions of the microbiome is to conduct microbiota manipulation experiments. Such experiments require reference stocks of bacteria and fungi isolates and specially designed model systems in which the functions of individual microbiome members can be tested in isolation or more general contributions of the microbiome to agroecosystem functioning can be investigated. This thesis addresses these research gaps by using next generation DNA sequencing to investigate how agricultural management practices affect soil and root microbiomes and, using a combination of microbial reference stocks and microcosm systems, outlines an approach to experimentally investigate the contributions of specific microbiome members and overall microbiome diversity to plant growth and overall agroecosystem functioning.

Using an agricultural field experiment near Zürich as a case study, Chapter 2 utilizes next generation DNA sequencing to investigate how four different cropping systems, including conventional management with intensive and without tillage and organic management with intensive and reduced tillage, influences the diversity of soil and root bacteria and fungi communities. Although the effects of the different cropping systems on bacteria and fungi richness were minimal, the different cropping systems had a marked effect on microbial

community composition. Soil bacterial communities were primarily structured by tillage, whereas soil fungal communities responded mainly to management type. In roots, management type was also the driving factor for bacteria, but fungi community composition was determined by changes in tillage intensity. Indicator species analysis was then performed to determine which bacteria and fungi taxa were responsive to the different cropping systems and their importance in a community context was assessed using co-occurrence network analysis. Overall, the results suggest that taxonomically diverse groups of microbes respond to agricultural practices, and, based on their abundance and level of co-occurrence with other community members, they may be important for determining community dynamics and microbiome functioning. Thus, the application of different cropping practices allows for the manipulation of potentially influential microbiome members.

Taxonomy based diversity surveys, like that conducted in Chapter 2, provide only limited information about a microbe's function in a community. Thus, discovering the microbial functions that could be targeted through microbiome manipulation requires model systems in which inoculation experiments can be conducted. The study in Chapter 3 presents published work that outlines an approach to investigate the composition and function of specific members of the root microbiome of *Trifolium pratense* (red clover), an important agricultural plant species, by combining culture independent and dependent approaches along with microcosm systems. The root bacteria microbiome of Trifolium was profiled using DNA sequencing, which revealed a high proportion of N-fixing rhizobia bacteria, in addition to other bacteria taxa, which may provide pathogen protection. Using standard microbiological techniques, a reference stock of 200 bacteria isolates was collected, which represented ~20% of the most abundant root community members based on comparisons of sequence similarity. A reductionist microcosm system was developed to explore root microbiome assembly under defined growth conditions and to conduct simplified microbiota inoculation experiments with plants. Four culturable members of the root bacteria microbiome were inoculated separately and in a simplified mini community, and the effects on plant growth were scored in three independent experiments. When inoculated alone, a *Flavobacterium* reduced plant growth, but this negative growth effect was alleviated when the bacterium was co-inoculated in the mini community. These results suggest that the negative effects of potential pathogens in the root microbiome can be alleviated in a more diverse community. Moreover, the presented experimental approach offers many opportunities for further experimentation with Trifolium and can be expanded to other agricultural plant species.

Microbes are known to contribute to a wide variety of ecosystem processes, and thus Chapter 4 employed a more holistic approach to manipulate general soil microbiome diversity and assess the effects on multiple ecosystem functions and overall multifunctionality. Chapter 2 demonstrated that agricultural management of soils can induce changes in the composition of bacteria and fungi communities in soil. To explore how such changes affect ecosystem functioning, strains from the bacteria reference stock (Chapter 3), as well as a collection of separately isolated fungi strains, were inoculated into an autoclaved sand/soil mixture with the goal of creating microbial communities dominated by bacteria, fungi, or a mix of both kingdoms. Autoclaved soil and non-autoclaved field soil served as a negative and positive controls, respectively, and the effects of these treatments on plant biomass production, litter decomposition, leaching volume, N-N_2O losses, and overall ecosystem multifunctionality were assessed in an experiment using a second set of larger, specially designed microcosms planted with a monoculture grassland. The inoculation success of the different bacteria and fungi isolates was assessed at the conclusion of the experiment using DNA amplicon sequencing. 69% of inoculated bacteria and 87% of inoculated fungi isolates mapped to an OTU sequence in the microcosm community profiles. However, inoculated isolates were also detected in microcosms receiving no microbial inoculum, possibly due to contamination, or an artefact of sterilization resistant DNA captured by the community sequencing. This resulted in no differences in bacteria or fungi richness in the different microbial treatments, with the exception of the positive control. However, the inoculation of isolates induced shifts in microbial community composition and influenced some of measured ecosystem functions. Notably, litter decomposition was 40% higher in the positive control treatment, highlighting the importance of a diverse microbial community in the decomposition process. Plant biomass production and overall ecosystem multifunctionality were also slightly lower in the mixed community treatment, possibly the result of the community composition. Overall, the results show that isolate collections and model experimental systems can be combined to explore microbial contributions to ecosystem functioning, but improvements to the microcosms can still be made.

The work presented here demonstrates that the abundance of potentially influential soil and root microbiome members can be managed through agricultural management practices. Microbiota manipulation experiments utilizing reference stocks of microbes and model experimental systems presented a promising approach for future investigations to elucidate the microbial functions that could be targeted to improve the sustainability of agriculture.

Zusammenfassung

Die Böden unsere Erde beherbergen eine enorme Artenvielfalt an Mikroorganismen einschliesslich Bakterien und Pilzen. Diese Diversität verdichtet sich im Innern, auf und um die Pflanzenwurzeln zu einem „hotspot“ von mikrobieller Aktivität. Die Gemeinschaften von Boden und Wurzel-assoziierten Mikroorganismen mitsamt ihrem Genpool funktionieren in ihrer Gesamtheit als Mikrobiom. Weil Mikroben eine Vielzahl von wichtigen Pflanzen- und Ökosystemfunktionen erfüllen – beispielsweise, Förderung des Pflanzenwachstums, Schutz vor Pathogenen oder Verbesserung der Nährstoffaufnahme – verspricht man sich, die Nachhaltigkeit der landwirtschaftlichen Produktion durch Ausnutzung solcher Mikrobiomeigenschaften erhöhen zu können. Dies ist besonders wichtig, da die moderne und intensive Landwirtschaft eine Vielzahl von Umweltproblemen wie beispielsweise hohe Nährstoffauswaschung, Emissionen von Treibhausgasen oder Verluste in Biodiversität mit sich bringt. Eine zukünftige Ausnutzung von Boden- und Wurzelmikrobiome, beispielsweise durch Fördern von Nützlingen oder Unterdrücken von Schädlingen, bedingt das Wissen, wie sich die mikrobiellen Gemeinschaften und ihre Funktionen durch landwirtschaftliche Bewirtschaftung steuern lassen. Nur wenige Studien habe Form der Bewirtschaftung (konventionell oder biologisch) kombiniert mit verschiedenen Pflugverfahren und deren Effekte auf Boden- und Wurzelmikrobiome untersucht. Zudem sind die Funktionen der meisten Mikroorganismen sind meist unbekannt. Eine vorgeschlagene Vorgehensweise um Funktionen von Mikrobiomen zu untersuchen, besteht im Aufbau von Stammsammlungen aus isolierten Mikroben, um diese in manipulativen Experimenten auf deren Funktion(en) testen zu können. Solche Experimente bedingen speziell entwickelte Modellsysteme, worin die Funktionen der einzelnen Mikrobiommitglieder separat oder im Gemeinschaftsverbund sowie deren Beiträge zu verschiedenen Ökosystemfunktionen getestet werden können. Diese Doktorarbeit adressierte diese Forschungslücken, um unter Einsatz von neuster DNS Sequenziertechnologie zu untersuchen, wie verschiedene Bewirtschaftungsarten die Boden- und Wurzelmikrobiome beeinflussen. Zudem zeigt diese Arbeit auf, dass manipulative Experimente in Mikrokosmen mit Isolaten aus Stammsammlungen nützlich sind, um die Beiträge einzelner Mikrobiommitglieder oder ganzer Mikrobiome auf das Pflanzenwachstum und das Funktionieren vom Gesamtökosystem untersuchen zu können.

Anhand eines landwirtschaftlichen Feldversuches in der Nähe von Zürich, wurde mittels moderner DNS Sequenziertechnologie untersucht, wie vier verschiedene Bewirtschaftungsysteme (konventionelle Bewirtschaftung mit oder ohne Pflug und

biologischer Landbau mit oder mit reduziertem Pflugeinsatz) die Diversität von Bakterien- und Pilzgemeinschaften im Boden und in der Wurzel beeinflussen (Kapitel 2). Obwohl der Effekt der verschiedenen Bewirtschaftungssysteme auf den Artenreichtum der Bakterien und Pilze nur minimal waren, hatten diese markante Effekte auf die Zusammensetzung der mikrobiellen Gemeinschaften. Während die Bakteriengemeinschaften im Boden hauptsächlich durch den Einsatz vom Pflug strukturiert werden, reagieren die Bodenpilze im Wesentlichen auf die Form der Bewirtschaftung. In Pflanzenwurzeln war die Form der Bewirtschaftung der treibende Faktor für die Zusammensetzung der Bakteriengemeinschaften während die Intensität des Pflügens die Pilzzusammensetzung bestimmt. Eine ‚Indikatorarten' Analyse wurde durchgeführt, um Bakterien und Pilze zu identifizieren, die spezifisch auf eines oder mehrere der verschiedenen Bewirtschaftungssysteme reagieren. Weiter wurde deren Bedeutung im Mikrobiom mittels Kookkurrenz Netzwerkanalyse untersucht. Die Resultate deuten darauf hin, dass taxonomisch vielfältige Gruppen von Mikroben jeweils auf die verschiedenen Bewirtschaftungssysteme reagieren und es sich dabei um wichtige Mikroben (basierend auf deren Häufigkeit und deren gemeinsamen Vorkommens mit anderen Gemeinschaftsmitgliedern) für die Zusammensetzung und Funktion der Mikrobiome handelt. Demnach können mittels verschiedener Bewirtschaftungsformen scheinbar einflussreiche Mikrobiommitglieder in ihren Häufigkeitsmustern manipuliert werden.

Taxonomie-basierte Diversitätsanalysen, wie jene in Kapitel 2, erlauben nur einen bedingten Einblick in die Funktionen von Mikroorganismen in einer Gemeinschaft. Die Suche nach Funktionen in Mikrobiomen, die sich manipulieren lassen, bedingt Modellsysteme worin funktionelle Inokulationsexperimente durchgeführt werden können. Die Studie in Kapitel 3 zeigt eine veröffentlichte Arbeit, die eine Vorgehensweise zur Untersuchung von Zusammensetzung und Funktion von spezifischen Mitgliedern des Wurzelmikrobioms der landwirtschaftlich wichtigen Pflanze *Trifolium pratense* (Rotklee) zeigt. Die Vorgehensweise kombiniert kultivierungsunabhängige und –abhängige Methoden einschliesslich Mikrokosmen als Modellsystem. Die Bakterien des Kleewurzelmikrobioms wurden mittels DNS Sequenzierung erfasst, was einen hohen Anteil von N-fixierenden Rhizobienbakterien zeigte und auch weitere Bakterien identifizierte, die vermutlich Schutz vor Pathogenen gewähren. Mit gewöhnlichen mikrobiologischen Kultivierungsmethoden, wurden 200 Bakterien isoliert und in einer Stammsammlung zusammengefasst, welche ~20% der häufigsten Wurzelbakterien entspricht. Ein reduktionistisches Modellsystem von Mikrokosmen wurde entwickelt, um die Ausbildung des Wurzelmikrobioms unter definierten Bedingungen zu erforschen und um Mikrobiotainokulierungsversuche mit Pflanzen durchführen zu können. Vier Mitglieder des

Kleewurzelmikrobioms wurden einzeln oder kombiniert als vereinfachte Minigemeinschaft inokuliert und die Effekte auf das Pflanzenwachstum in drei unabhängigen Experimenten bestimmt. Während ein *Flavobakterium* bei Einzelinokulation das Pflanzenwachstum hemmte, so war dieser negative Effekt aufgehoben, wenn das Bakterium in einer Minigemeinschaft inokuliert wurde. Diese Beobachtung deutet darauf hin, dass negative Effekte von möglichen Pathogenen im Gemeinschaftskontext von einem diverseren Wurzelmikrobiom abgeschwächt werden. Das entwickelte Modelsystem offeriert nun unzählige Möglichkeiten das Wurzelmikrobiom von Trifolium weiter zu untersuchen und kann zudem auf weitere landwirtschaftlich genutzte Pflanzen ausgeweitet werden.

Mikroorganismen sind bekannt, dass sie zu einer breiten Palette von Ökosystemprozessen beitragen. Für Kapitel 4 wurde ein ganzheitlicherer Ansatz gewählt wo die generelle Diversität des Bodenmikrobioms manipuliert wurde, um die Effekte auf verschiedene Ökosystemleistungen und die Multifunktionalität des ganzen Ökosystems zu untersuchen. Kapitel 2 zeigte, dass Bodenbewirtschaftung Veränderungen in der Zusammensetzung der Bakterien- und Pilzgemeinschaften im Boden zur Folge hat. Um nun zu untersuchen, wie solche Veränderungen das Funktionieren des Agrarökosystems beeinflussen, wurden Bakterien der Stammsammlung aus Kapitel 3 wie auch zusätzlich isolierte Pilze in Modellsystemen mit einer autoklavierten Sand/Erde-Mischung zugegeben. Dabei wurden mikrobielle Bodengemeinschaften etabliert, die entweder von Bakterien oder Pilzen oder einer Mischung beider Domänen dominiert waren und die zusätzlichen Verfahren mit Zugabe von autoklavierter Erde oder natürlicher Felderde dienten als Negativ- und Positivkontrollen. Diese Verfahren wurden in einem zweiten, etwas grösseren Modellsystem, bepflanzt mit dem Gras *Lolium perenne*, auf Effekte auf Pflanzenbiomasse, Abbau von Pflanzenresten, Auswaschung, Stickstoffverluste, Lachgasemissionen und die Multifunktionalität des Ökosystems hin untersucht. Am Ende des Experimentes wurde der Inokulierungserfolg der verschiedenen Bakterien und Pilzisolate mittels DNS Sequenzierung bestimmt. 69% der inokulierten Bakterien und 87% der inokulierten Pilze stimmten mit Sequenzgruppen (OTU, Englisch für „operational defined taxonomic unit“) überein, die in den Bodenmikrobiomprofilen der Mikrokosmen gefunden wurden. Sequenzen der inokulierten Isolate wurden jedoch auch in Mikrokosmen gefunden, in die kein Inokulum zugegeben worden war, und dies wurde als mögliche Kontamination oder als Artefakt von DNS, die nach Sterilisation weiterhin vorlag, erklärt. Mit Ausnahme der Positivkontrolle, ergaben sich keine Unterschiede in der Artenvielfalt von Bakterien und Pilzen in den verschiedenen Verfahren. Die inokulierten Isolate veränderten jedoch die Zusammensetzung der mikrobiellen Gemeinschaften und

beeinflussten so einige der gemessenen Ökosystemleistungen. Der Abbau von Pflanzenresten war beispielsweise 40% höher in den Positivkontrollen, was die Bedeutung von artenreichen mikrobiellen Gemeinschaften in Abbauprozessen herausstreicht. Auch in Abhängigkeit der Zusammensetzung der Bodenmikrobiota wurden eine leicht reduzierte Pflanzenproduktivität und Multifunktionalität im gemischt inokulierten Verfahren gefunden. Obwohl Mikrokosmenexperimente noch Verbesserungspotential haben, zeigen die ersten Resultate, dass Mikrobiotamanipulationen in Mikrokosmen dienlich sind, um den Beitrag von Mikroorganismen zum Funktionieren von Ökosystemen, zu erforschen.

Die hier vorliegende Arbeit zeigt, dass potentiell wichtige Boden- und Wurzelmikrobiommitglieder durch gezielte landwirtschaftliche Bewirtschaftung in ihrer Häufigkeit gesteuert werden können. Die manipulativen Experimente mit Mikroorganismen aus Stammsammlungen lieferten den Eignungsnachweis, um zukünftig die exakten Funktionen dieser „bewirtschaftbaren“ Boden- und Wurzelmikrobiommitglieder untersuchen zu können. Fernziel wäre die Nachhaltigkeit der Landwirtschaft durch gezieltes Steuern der *Funktionen* von Boden- und Wurzelmikrobiomen zu verbessern.

Chapter 1:

General Introduction

The biodiversity of soil

Soil is a dynamic and complex biological system, and it has been estimated that soils contain one quarter to one third of all life on earth [1,2]. This life exists in a wide range of shapes and sizes, from large macro-fauna like ants and earthworms to smaller meso-fauna like mites or collembola. At the microscopic scale are the microbes like bacteria, fungi, archaea and viruses which represent a large portion of the living biomass on earth [3]. All together, these microbes and their associated genes collectively function as a microbiome, but it is thought that >90% of all life in soil is bacteria and fungi [4]. Bacteria diversity in soil has been estimated to be 6,000-38,000 taxa per gram of soil with billions of individual bacteria cells [5]. Fungi are typically less abundant than bacteria in soils, but fungi diversity estimates can still be as high as 200 different taxa with hundreds of meters of fungal hyphae in a single gram of soil [6].

The specific functions of some soil bacteria and fungi are well documented. For example, some bacteria belonging to the family Rhizobiaceae associate with the roots of legumes, where they fix atmospheric nitrogen (N) into plant available ammonium [7]. Arbuscular mycorrhizal fungi (AMF) are well known for their symbiotic relationship with many terrestrial plants. In exchange for carbon, they provide their hosts with phosphorus (P). The provision of N and P by microbes is an important determinant of plant productivity because N and P, along with potassium, are the main elements limiting plant productivity in terrestrial ecosystems [8]. However, the functions of most fungal and bacterial taxa and their complex interactions with soil and plants are still hidden within the "black box" [9], and ascribing specific ecosystem processes to microorganisms remains a major challenge in microbial ecology [10].

The rhizosphere is a "hot spot" of microbial activity

Despite the large amount of soil microbial diversity, this microbial life only occupies a small area of the available soil volume, congregating in so-called microbial "hot spots." These hot spots are defined as small microhabitats within the soil volume where physiochemical properties differ from that of the surrounding soil and microbial activity and interactions are increased [2,11]. One such microbial hot spot comprises the soil surrounding the roots of plants, known as the rhizosphere. Originally, the concept of the rhizosphere was described by Hiltner in 1904 as the zone of soil immediately adjacent to a plant's roots [12]. More recently, however,

the rhizosphere concept has been expanded to include the ectorhizosphere – a more specific term for the original definition -, the rhizoplane - the surface of the root -, and the endosphere – the inner root tissues- that are colonized by microbes [13] (Fig. 1). Collectively, the microbiota and their associated genes inhabiting these rhizosphere compartments are termed the root microbiome. The richness and composition of root microbial communities is typically different from that of the surrounding bulk soil [14,15]. In contrast to taxonomically more diverse bulk soil communities, root bacteria communities are typically dominated by the major bacteria phyla *Proteobacteria, Actinobacteria,* and *Bacteroidetes* [16]. With the exception of AMF, less work has focused on characterizing root fungi communities compared to bacteria [17]. However, the root-associated fungi communities generally consist of saprophytic yeasts and filamentous fungi from the *Ascomycota* and *Basidiomycota* [18]. A number of plant growth promoting, as well as pathogenic, fungi are also known to colonize the different compartments of the rhizosphere [19].

The colonization and subsequent composition of root-associated bacteria and fungi communities is determined by a combination of biotic, abiotic, edaphic and plant-host factors [20]. The bulk soil microbial community is considered to be the starting pool from which the plant actively recruits microbes to its roots. Thus, soil type and, in turn, the composition of the microbial community in the soil in which plants root is an important factor determining the composition of their root bacteria [14,21,22] and fungi [23] communities. While many environmental factors have been identified as contributing to the composition of microbial communities in bulk soil [2], soil pH has been identified as major determinant of bacteria community composition [24–26]. Differences in soil pH have also been identified as a driver of soil fungi community composition [27], but differences in soil nutrients, primarily extractable P and the soil carbon (C):N ratio, also play an important role [26].

As these edaphic factors exert influence on the community composition of bulk soil microbial communities, so too do plants exert influence on their root microbiome through the rhizodeposition of complex carbon and antimicrobial compounds, mucilage, sloughed root border cells, and various plant signaling hormones, all which create ideal conditions for microbial growth [20,28] (Fig. 1). This release of root exudates acts as a first selection step to attract a subset variety of soil microbes to the ectorhizosphere. In a second step, host-plant genetic factors mediate molecular signals that attract a subset of microbes from the ectorhizosphere to bind to the rhizoplane and subsequently a subset of the rhizoplane community to enter and proliferate in the endosphere [20,22] (Fig. 1).

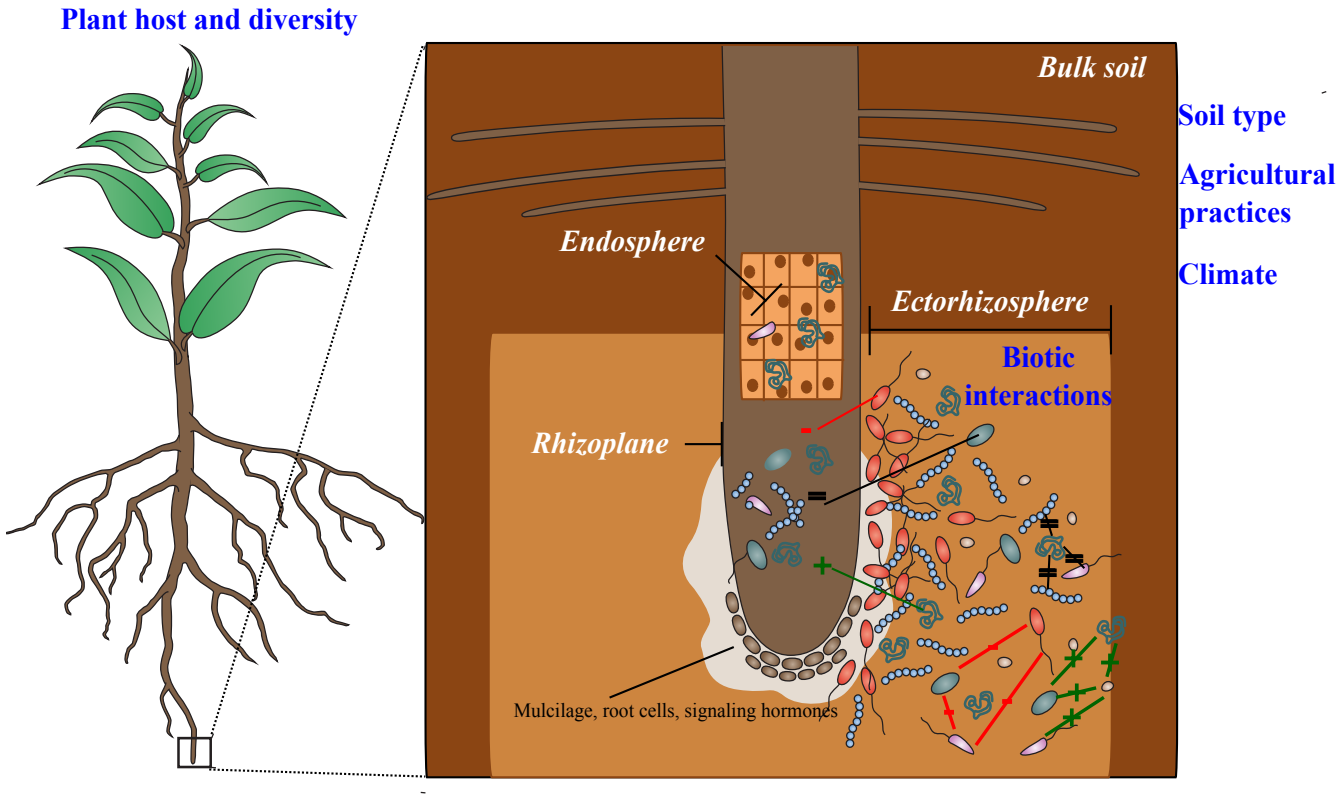

Figure 1: Processes of rhizosphere microbial community assembly. The compositions of the different compartments of the soil and root microbiome (indicated in white) are influenced by a number of plant-host, biotic, and abiotic factors (in blue). Microbes in these compartments can have positive (green), negative (red) or neutral (black) interactions with other community members and their plant host. The number of microbes in each compartment represents a progressive reduction in microbial richness and diversity in the rhizoplane and endosphere compartments [14]. Figure modified from Philippot *et al.,* [29].

The bacterial and fungal members of the root microbiome can establish beneficial, detrimental, or neutral associations with their host and are one of the key determinants of plant health and productivity [30,31] (Fig. 1). For example, a number of rhizobacteria possess biocontrol abilities that can help plants to suppress diseases by inducing plant resistance or outcompeting pathogens for nutrients and niche space. Other bacteria can directly stimulate plant growth through the production of hormones [32]. Similarly, a number of endophytic root fungi have been shown to confer tolerance to heat and salinity to their hosts, increase root and shoot biomass, and protect their hosts against pathogens [19]. With this in mind, gaining a better understanding of how plants assemble a root microbiome, its composition, and elucidating the functions of members is a rational step in the eventual manipulation of the root microbiome for plant beneficial traits that could, for example, increase agricultural productivity and sustainability [33].

Agricultural effects on soil and root communities

The need to increase the sustainability of agriculture has been identified as a major research area in order to reduce the environmentally harmful effects of modern, intensive agriculture [34], which include nutrient losses due to leaching and greenhouse gas production from over use of fertilizers, increased soil erosion, and biodiversity losses [35]. A number of practices improve the sustainability of agriculture, including organic farming [36] and reduced or no-tillage [37]. These practices aim to enhance soil fertility while maintaining crop yields through supporting diverse and active microbial communities [38]. Harnessing the power of microbes to improve the sustainability of agriculture is logical because soil bacteria and fungi and play important roles in a number of important agroecosystem functions, including decomposition, nutrient cycling, protection against pathogens, and plant growth promotion [39]. As a result, the diversity of belowground microbial communities largely determines the productivity of agricultural ecosystems [40].

An important question about the relationship between soil microbes and agroecosystem functioning is how ecosystem processes will change if bacteria and fungi communities change through disturbance. This is especially applicable to agriculture where the physical and biological properties of the soil are altered due to management practices [41]. Application of organic and inorganic fertilizers can result in new microbial inputs, change soil chemistry, nutrient availability, and consequently induce shifts in bacterial and fungal communities [42–44]. Intensive plowing of fields can break up macro and micro aggregates within soil, reducing habitats for both bacteria and fungi [45]. Soil tillage can also destroy hyphal networks and lead to a decline in abundance of not only general soil fungi [46], but also induce shifts in bulk soil [47,48] and endophytic AMF communities [49]. Furthermore, the effects of chemicals, such as fungicides, herbicides, and insecticides are often not only limited to pests, but can have detrimental effects on beneficial or other non-target soil microbes [50,51].

The abovementioned studies demonstrate that agricultural management of soils generally affects soil microbial communities, but such effects may depend on the microbial kingdom being studied and the different farming systems being compared [50,52]. Moreover, given the bulk soil microbiome's influence on the composition of a plant's root microbiome [53], it is possible that effects of agricultural practices on soil microbial communities also result in differences in the root microbiome [54]. Recent work has characterized the root microbiomes of a number of important crop species like soybean [55], maize [56], rice [22], barley [57], sugarcane [58], and grapevine [59]. However, these studies did not specifically examine how root communities respond to different cropping systems or tillage practices, and therefore

further research is required to improve our understanding of the effects of agriculture on the composition and function of soil and root microbial communities. Such work has been identified as a necessity for identifying specific agronomic practices that may improve plant growth, enhance soil fertility, and improve the overall sustainability of agriculture [60].

Methods to characterize microbial communities

Investigating soil and root microbial communities and how they respond to agricultural management requires tools that can estimate diversity and characterize community composition. These tools are considered to be either culture dependent or independent. Traditionally, analysis of microbial communities was conducted using culture dependent techniques in which soil or root samples are shaken or blended with a sterile buffer and serially diluted. Samples of these dilutions are plated on a variety of culture media, and diversity can be estimated using plate count methods [61]. However, there are a number of drawbacks to culture dependent methods, which include laborious procedures [2], biased diversity estimates based on the culture media used [62], and the estimate that >99% of soil microbes cannot be cultured [63].

Because of the limitations of culture-based methods, many microbial community analyses are now conducted using culture independent molecular methods, which involve the extraction of genomic deoxyribonucleic acid (DNA) from soil and root samples and amplification of a marker gene sequence using polymerase chain reaction (PCR) [64]. For bacteria community profiling, the 16S rRNA gene is often used; whereas fungal community profiling often relies on amplification of one or both of the internal transcribed spacer (ITS) regions [65]. Recent advances in next generation sequencing (NGS) technology, such as Illumina's MiSeq platform, means that millions of DNA sequences per PCR reaction can be generated, and amplicon libraries from many environmental samples can be pooled together in a single sequencing run [66]. This multiplexing of samples is achieved through the use of barcoded PCR primers which append a unique oligonucleotide sequence to amplicons from each sample [67]. After sequencing, reads are demultiplexed and assigned back to their original samples and quality filtered using a suite of bioinformatics tools. Subsequently, the high quality sequences are binned into operational taxonomic units (OTU) based on sequence similarity (typically 97% for species level) and a representative sequence chosen for each OTU. A taxonomy assignment can be assigned to these representative OTUs using a number of available taxonomy databases, and a table of sequence counts per OTU is generated for downstream data analysis [65]. This analysis can include exploration of alpha and beta

diversity [68], multivariate statistical testing of hypotheses about the effects of collected environmental data on microbial community structure [69], identification of indicator microbial taxa [70], or network analyses to explore co-occurrence patterns between OTUs and identify potentially influential community members [71,72]. Together, these techniques provide valuable insights as to how microbial communities change across space, time, or respond to experimental treatments [73].

Towards functional investigations of bacteria and fungi communities

Sequencing of 16S rRNA gene and ITS amplicon libraries is a valuable tool for describing the taxonomic and, in the case of the 16S rRNA gene, phylogenetic diversity of soil and root microbiomes and determining how the composition of these communities responds to biological, environmental, or experimental factors. However, community profiling of microbial communities provides little information about the function of its members because it is notoriously problematic to infer a microbe's function from a taxonomy assignment [33,74]. Therefore, more direct approaches are required. Other molecular methods for elucidating microbial function have been suggested, such as "-omics" techniques like metagenomics, metatranscriptomics, and metaproteomics; however, there are a number of drawbacks to these methods, including the high number of biological replicates required, low sequencing coverage, and the time and financial investment required [75]. As a result, a return to a more "traditional" approach of using microbiological methods to build reference stocks of bacteria and fungi isolates for use in microbiome manipulation experiments has recently been suggested [33]. Although culture dependent methods also suffer from a number of previously discussed drawbacks, previous works have employed combinations of culture dependent and independent techniques to quantify the culturable fraction of the root bacteria microbiomes of maize [76], and more recently, *Arabidopsis thaliana* [77]. The work of Bai *et al.,* [77] is a particularly promising example demonstrating that ~60% of the abundant root bacteria community is culturable. However, there are still many opportunities to isolate reference stocks of microbes from other plant species to and experimentally investigate microbial contributions to plant and overall agroecosystem functioning.

Translating the insights gained from NGS projects into potential benefits for plants and agroecosystem functioning requires experiments in which the presence or abundance of specific microbial taxa or general microbial diversity are manipulated. Such studies can take two different approaches: a reductionist approach, in which contributions of a specific species or group of microbes to ecosystem processes are investigated in isolation, or a holistic

approach, in which more natural, complex microbial communities are used to test their impact on a variety of ecosystem responses [9]. Conducting these functional experiments requires a contained system in which general microbial diversity or the presence/absence of specific microbes can be manipulated without contamination from the outside and data on the effects on plant and ecosystem functioning can be collected and assessed [40]. Recent works have utilized specially designed microcosm systems to investigate the contributions of general soil biodiversity to overall ecosystem functioning [78,79], the roles of AMF and rhizobia in legume [80] and overall plant productivity [81], or inoculated microbial reference stock members into microcosms to examine the processes of root microbiome assembly [77,82]. Thus, microcosm systems are a widely used and useful tool. Moreover, when combined with the power of NGS technology and reference stocks of bacteria and fungi isolates, a unique opportunity exists in which agricultural effects on soil and root microbial communities can be investigated and its consequences for plant and overall agroecosystem functioning can begin to be tested in reductionist and holistic experimental systems.

Thesis outline

The aim of this PhD work was to determine how agricultural management practices affect soil and root bacteria and fungi communities and, using a combination of microbial reference stocks and microcosm systems, begin to experimentally investigate the contributions of specific microbiome members and overall microbiome diversity to plant growth and overall agroecosystem functioning. My original research is documented in this thesis in the following three chapters (Fig. 2). In Chapter 2, I used an agricultural field trial as a case study to assess the effects of agricultural management on soil and root microbial communities and explore the potential to manipulate these communities using different cropping practices (Fig. 2a). The Farming Systems and Tillage (FAST) trial was established near Zürich to compare the four main cropping systems in Switzerland, namely conventional management with intensive and without tillage and organic management with intensive and reduced tillage [83]. While management practices like organic agriculture or no- and reduced- tillage systems are often promoted for their reduced environmental impact, their yields are often lower that in conventional agriculture [84,85]. Recently, Wittwer *et al.,* [83] investigated the effects of the different cropping systems on winter wheat and maize yields. However, the effects of management types and different tillage intensities on belowground communities at the site have not been explored. Thus, I investigated the impact of these four cropping systems on soil and root bacterial and fungal communities in winter wheat using culture independent NGS. More

specifically, I asked if soil and root microbial communities respond similarly to the different cropping practices. Additionally, I identified which specific members of the soil and root microbiome respond to the different cropping practices and assessed their potential importance for the overall community using co-occurrence network analysis.

Chapters 3 and 4 lay the groundwork for functional examinations of the importance of root and soil microbial communities for plant and overall ecosystem functioning. In Chapter 3, I first combined culture independent and dependent methods to characterize the composition of the root bacteria microbiome of red clover, an important agricultural plant species (Fig. 2b) that is typically used (together with ryegrass) in crop rotations in Switzerland, including the FAST trial investigated in Chapter 2. I presented a reference stock of 200 bacteria isolates, cultured from red clover roots, and examined the overlap between the reference stock and NGS-generated community profiles to estimate the culturable fraction of the plant's root bacteria microbiome. In a second step, I presented a specially designed, reductionist microcosm system and move towards functional investigations of specific root bacteria microbiome members by inoculating selected bacteria from the reference stock collection, both individually and in a simplified mini community, into the microcosms. I assessed the effects of these bacteria inoculation treatments on plant biomass production in three replicate experiments.

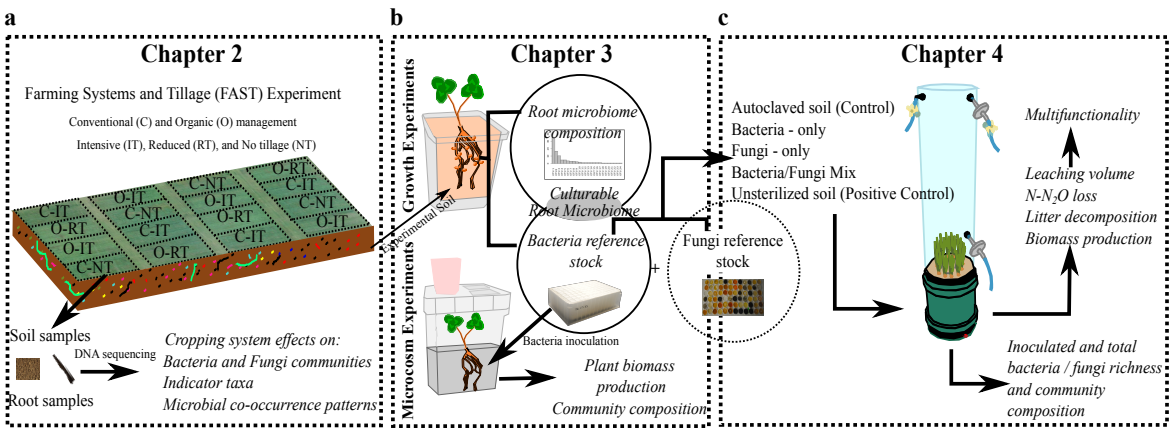

Figure 2: Schematic overview of the dissertation structure. The major points explored within each chapter are indicated in italics. Arial photo of FAST trial in **(a)** modified from one provided by Raphäel Wittwer.

In Chapter 4, I take a more holistic approach to examine the consequences of manipulating soil bacteria and fungi communities on a number of ecosystem functions and overall ecosystem multifunctionality (Fig. 2c)**.** Previous work, including the results presented in Chapter 2 of this thesis, have shown that agricultural management of soils can result in shifts in soil microbial community composition. Agricultural management of soils can reduce

belowground microbial diversity, the complexity of the total soil food web [38,86,87], and can result in shifts in soil fungi:bacteria ratios [88–90]. Bacteria and fungi comprise the majority of microbial biomass in soils [4] and often have distinct functions with regard to decomposition and nutrient cycling [91]. Thus, it is important to investigate how soil ecosystem functioning will change if soil bacteria and fungi community richness and composition is manipulated. I utilized the bacteria reference stock from Chapter 3 and, additionally, built a reference stock of approximately 200 fungi strains. I inoculated a diverse community of the representative strains of the bacteria and fungi of the reference stocks into autoclaved microcosm soils. The goal was to create different microbial treatments dominated by bacteria, fungi, or a mix of microbes from both kingdoms, as well as non-inoculated and unsterilized field soil. This experiment was conducted in a second set of larger, specially designed microcosms in which incoming air and water is filtered to prevent outside contamination and planted with monoculture grassland. The design of these microcosms allows for multiple ecosystem functions to be assessed simultaneously, and thus I quantified the effects of the different microbial treatments on plant biomass production, plant litter decomposition, N losses due to N_2O production, leaching volume, and overall ecosystem functioning by calculating an ecosystem multifunctionality index.

Overall, the results presented here show that agricultural management can have marked, yet contrasting, effects on the soil and root microbiome and may effect highly influential community members. My experimental work demonstrates how reference stocks of microbial isolates can pave the way for experiments in which the presence of specific microbiome members or changes in the composition of the entire community could be manipulated in microcosms. Such experiments are important for determining the function of microbiome members both individually and in a community context and determining how we can capitalize on the power of the microbiome to improve agroecosystem functioning.

References

1. Orgiazzi A, Bardgett R, Barrios E, Behan-Pelletier V, Briones M, Chotte J-L, et al. Global Soil Biodiversity Atlas. Luxembourg: Publications Office of the European Union; 2016.

2. Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G. Microbial diversity and soil functions. Eur. J. Soil Sci. 2003;54:655–70.

3. Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: the unseen majority. Proc Natl Acad Sci U S A. 1998;95:6578–83.

4. Six J, Frey SD, Thiet RK, Batten KM. Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems. Soil Sci. Soc. Am. J. 2006;70:555.

5. Curtis TP, Sloan WT, Scannell JW. Estimating prokaryotic diversity and its limits. Proc. Natl. Acad. Sci. U. S. A. 2002;99:10494–9.

6. Bardgett RD, van der Putten WH. Belowground biodiversity and ecosystem functioning. Nature. 2014;515:505–11.

7. Oldroyd GED, Murray JD, Poole PS, Downie JA. The rules of engagement in the legume-rhizobial symbiosis. Annu. Rev. Genet. 2011;45:119–44.

8. Chapin FS. The Mineral Nutrition of Wild Plants. Annu. Rev. Ecol. Syst. 1980;11:233–60.

9. Cortois R, De Deyn GB. The curse of the black box. Plant Soil. 2011;350:27–33.

10. Torsvik V, Øvreås L. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol. 2002;5:240–5.

11. Kuzyakov Y, Blagodatskaya E. Microbial hotspots and hot moments in soil: Concept & review. Soil Biol. Biochem. 2015;83:184–99.

12. Hartmann A, Rothballer M, Schmid M. Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. Plant Soil. 2008;312:7–14.

13. Morgan JAW, Bending GD, White PJ. Biological costs and benefits to plant-microbe interactions in the rhizosphere. J. Exp. Bot. 2005;56:1729–39.

14. Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, et al. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature. 2012;488:91–5.

15. Jumpponen A, Johnson LC. Can rDNA analyses of diverse fungal communities in soil and roots detect effects of environmental manipulations--a case study from tallgrass prairie. Mycologia. 2005;97:1177–94.

16. Hacquard S, Garrido-Oter R, González A, Spaepen S, Ackermann G, Lebeis S, et al. Microbiota and host nutrition across plant and animal kingdoms. Cell Host Microbe. 2015;17:603–16.

17. Costa R, Götz M, Mrotzek N, Lottmann J, Berg G, Smalla K. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. FEMS Microbiol. Ecol. 2006;56:236–49.

18. Bueé M, De Boer W, Martin F, van Overbeek L, Jurkevitch E. The rhizosphere zoo: An overview of plant-associated communities of microogramisms, including phages, bacteria, archaea, and fungi, and some of their structuring factors. Plant Soil. 2009;321:189–212.

19. Rodriguez RJ, White JF, Arnold AE, Redman RS. Fungal endophytes: diversity and functional roles. New Phytol. 2009;182:314–30.

20. Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 2013;64:807–38.

21. Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, et al. Defining the core Arabidopsis thaliana root microbiome. Nature. 2012;488:86–90.

22. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, et al. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc. Natl. Acad. Sci. 2015;112:E911–20.

23. Bonito G, Reynolds H, Robeson MS, Nelson J, Hodkinson BP, Tuskan G, et al. Plant host and soil origin influence fungal and bacterial assemblages in the roots of woody plants. Mol. Ecol. 2014;23:3356–70.

24. Fierer N, Jackson RB. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. U. S. A. 2006;103:626–31.

25. Lauber CL, Hamady M, Knight R, Fierer N. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Appl. Environ. Microbiol. 2009;75:5111–20.

26. Lauber CL, Strickland MS, Bradford MA, Fierer N. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol. Biochem. 2008;40:2407–15.

27. Rousk J, Bååth E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, et al. Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J. 2010;4:1340–51.

28. Turner TR, James EK, Poole PS. The Plant Microbiome. Genome Biol. 2013;14:209.

29. Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH. Going back to the roots: the microbial ecology of the rhizosphere. Nat. Rev. Microbiol. 2013;11:789–99.

30. Berg G, Grube M, Schloter M, Smalla K. Unraveling the plant microbiome: Looking back and future perspectives. Front. Microbiol. 2014;5:1–7.

31. Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y. The rhizosphere: A playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil. 2009;321:341–61.

32. Lugtenberg B, Kamilova F. Plant-Growth-Promoting Rhizobacteria. Annu. Rev. Microbiol. 2009;63:541–56.

33. Schlaeppi K, Bulgarelli D. The Plant Microbiome at Work. Mol. Plant Microbe Interact. 2015;28:212–7.

34. Tilman D. Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices. Proc. Natl. Acad. Sci. U. S. A. 1999;96:5995–6000.

35. Matson P, Parton W, Power A, Swift M. Agricultural Intensification and Ecosystem Properties. Science. 1997;277:504–9.

36. Reganold JP, Wachter JM. Organic agriculture in the twenty-first century. Nat. Plants. 2016;2:15221.

37. Hobbs PR, Sayre K, Gupta R. The role of conservation agriculture in sustainable agriculture. Philos. Trans. R. Soc. London B Biol. Sci. 2008;363:543–55.

38. Mäder P, Fliessbach A, Dubois D, Gunst L, Fried P, Niggli U. Soil fertility and biodiversity in organic farming. Science. 2002;296:1694–7.

39. Brussaard L, Behan-Pelletier V, Bignell D, Brown V, Didden W, Folgarait P, et al. Biodiversity and ecosystem functioning in soil. Ambio. 1997;26:563–70.

40. van der Heijden MGA, Bardgett RD, Van Straalen NM. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol. Lett. 2008;11:296–310.

41. Jangid K, Williams MA, Franzluebbers AJ, Sanderlin JS, Reeves JH, Jenkins MB, et al. Relative impacts of land-use, management intensity and fertilization upon soil microbial community structure in agricultural systems. Soil Biol. Biochem. 2008;40:2843–53.

42. Bardgett RD, Lovell RD, Hobbs PJ, Jarvis SC. Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biol. Biochem. 1999;31:1021–30.

43. Schneider S, Hartmann M, Enkerli J, Widmer F. Fungal community structure in soils of conventional and organic farming systems. Fungal Ecol. 2010;3:215–24.

44. Hartmann M, Frey B, Mayer J, Mäder P, Widmer F. Distinct soil microbial diversity under long-term organic and conventional farming. ISME J. 2014;9:1177–1194.

45. Strickland MS, Rousk J. Considering fungal:bacterial dominance in soils – Methods, controls, and ecosystem implications. Soil Biol. Biochem. 2010;42:1385–95.

46. Jackson LE, Calderon FJ, Steenwerth KL, Scow KM, Rolston DE. Responses of soil microbial processes and community structure to tillage events and implications for soil quality. Geoderma. 2003;114:305–17.

47. Verbruggen E, Röling WFM, Gamper HA, Kowalchuk GA, Verhoef HA, van der Heijden MGA. Positive effects of organic farming on below-ground mutualists : large-scale comparison of mycorrhizal fungal communities in agricultural soils. New Phytol. 2010;186:968–79.

48. Säle V, Aguilera P, Laczko E, Mäder P, Berner A, Zihlmann U, et al. Impact of conservation tillage and organic farming on the diversity of arbuscular mycorrhizal fungi. Soil Biol. Biochem. 2015;84:38–52.

49. Jansa J, Mozafar A, Kuhn G, Anken T, Ruh R, Sanders IR, et al. Soil tillage affects the community structure of mycorrhizal fungi in maize roots. Ecol. Appl. 2003;13:1164–76.

50. Bünemann EK, Schwenke GD, Zwieten L Van. Impact of agricultural inputs on soil organisms—a review. Aust. J. Soil Res. 2006;44:379.

51. Miller RM, Lodge DJ. Fungal Responses to Disturbance : Agriculture and Forestry. In: DT W, DT S, editors. Mycota IV Environ. Microb. Relationships. Springer Berlin Heidelberg; 1997. p. 65–84.

52. Postma-Blaauw M, Goede R de, Ecology S, February N, Jack H. Soil biota community structure and abundance under agricultural intensification and extensification. Ecology. 2010;91:460–73.

53. Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A. The importance of the microbiome of the plant holobiont. New Phytol. 2015;206:1196–206.

54. Seghers D, Wittebolle L, Top EM, Verstraete W, Siciliano S. Impact of agricultural practices on the Zea mays L. endophytic community. Appl. Environ. Microbiol. 2004;70:1475–82.

55. Mendes LW, Kuramae EE, Navarrete A a, van Veen J a, Tsai SM. Taxonomical and functional microbial community selection in soybean rhizosphere. ISME J. 2014;8:1577–87.

56. Peiffer JA, Spor A, Koren O, Jin Z, Tringe SG, Dangl JL, et al. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. U. S. A. 2013;110:6548–53.

57. Bulgarelli D, Garrido-Oter R, Münch PC, Weiman A, Dröge J, Pan Y, et al. Structure and function of the bacterial root microbiota in wild and domesticated barley. Cell Host Microbe. 2015;17:392–403.

58. Yeoh YK, Paungfoo-Lonhienne C, Dennis PG, Robinson N, Ragan MA, Schmidt S, et al. The core root microbiome of sugarcanes cultivated under varying nitrogen fertiliser application. Environ. Microbiol. 2015;18:1338–51.

59. Zarraonaindia I, Owens SM, Weisenhorn P, West K, Hampton-Marcell J, Lax S, et al. The soil microbiome influences grapevine-associated microbiota. MBio. 2015;6:e02527-14.

60. Busby PE, Soman C, Wagner MR, Friesen ML, Kremer J, Bennett A, et al. Research priorities for harnessing plant microbiomes in sustainable agriculture. PLOS Biol. 2017;15:e2001793.

61. Hill GT, Mitkowski NA, Aldrich-Wolfe L, Emele LR, Jurkonie DD, Ficke A, et al. Methods for assessing the composition and diversity of soil microbial communities. Appl. Soil Ecol. 2000;15:25–36.

62. Tabacchioni S, Chiarini L, Bevivino A, Cantale C, Dalmastri C. Bias caused by using different isolation media for assessing the genetic diversity of a natural microbial population. Microb. Ecol. 2000;40:169–76.

63. Hugenholtz P, Pace NR. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. Trends Biotechnol. 1996;14:190–7.

64. Kent AD, Triplett EW. Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu. Rev. Microbiol. 2002;56:211–36.

65. Goodrich JK, Di Rienzi SC, Poole AC, Koren O, Walters WA, Caporaso JG, et al. Conducting a Microbiome Study. Cell. 2014;158:250–62.

66. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J.; 2012;6:1621–4.

67. Binladen J, Gilbert MTP, Bollback JP, Panitz F, Bendixen C, Nielsen R, et al. The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. PLoS One. 2007;2:1–9.

68. Lozupone CA, Knight R. Species divergence and the measurement of microbial diversity. FEMS Microbiol. Rev. 2008;32:557–78.

69. Ramette A. Multivariate analyses in microbial ecology. FEMS Microbiol. Ecol. 2007;62:142–60.

70. de Cáceres M, Legendre P. Associations between species and groups of sites: indices and statistical inference. Ecology. 2009;90:3566–3574.

71. van der Heijden MGA, Hartmann M. Networking in the Plant Microbiome. PLOS Biol. 2016;14:e1002378.

72. Agler MT, Ruhe J, Kroll S, Morhenn C, Kim ST, Weigel D, et al. Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. PLoS Biol. 2016;14:e1002352.

73. Barberan A, Bates ST, Casamayor EO, Fierer N. Using network analysis to explore co-occurrence patterns in soil microbial communities. ISME J. 2012;6:343–51.

74. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 2013;31:814–21.

75. Knight R, Jansson J, Field D, Fierer N, Desai N, Fuhrman J a, et al. Unlocking the potential of metagenomics through replicated experimental design. Nat. Biotechnol. 2012;30:513–20.

76. Chelius MK, Triplett EW. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. 2001;41:252–63.

77. Bai Y, Müller DB, Srinivas G, Garrido-Oter R, Potthoff E, Rott M, et al. Functional overlap of the Arabidopsis leaf and root microbiota. Nature. 2015;528:364–9.

78. Wagg C, Bender SF, Widmer F, van der Heijden MGA. Soil biodiversity and soil community composition determine ecosystem multifunctionality. Proc. Natl. Acad. Sci. U. S. A. 2014;1–5.

79. Pellkofer S. Soil Biodiversity and Ecosystem Functioning. University of Zürich; 2015.

80. van der Heijden MG, de Bruin S, Luckerhoff L, van Logtestijn RS, Schlaeppi K. A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity, plant nutrition and seedling recruitment. ISME J. 2016;10:389–99.

81. van der Heijden MGA, Bakker R, Verwaal J, Scheublin TR, Rutten M, van Logtestijn R, et al. Symbiotic bacteria as a determinant of plant community structure and plant productivity in dune grassland. FEMS Microbiol. Ecol. 2006;56:178–87.

82. Haney CH, Samuel BS, Bush J, Ausubel FM. Associations with rhizosphere bacteria can confer an adaptive advantage to plants. Nat. Plants. 2015;1:1–9.

83. Wittwer RA, Dorn B, Jossi W, van der Heijden MGA. Cover crops support ecological intensification of arable cropping systems. Sci. Rep. 2017;7:10.1038/srep41911.

84. Seufert V, Ramankutty N, Foley JA. Comparing the yields of organic and conventional agriculture. Nature. 2012;485:229–32.

85. Pittelkow CM, Liang X, Linquist B a., van Groenigen KJ, Lee J, Lundy ME, et al. Productivity limits and potentials of the principles of conservation agriculture. Nature. 2014;517:365–7.

86. de Vries FT, Thebault E, Liiri M, Birkhofer K, Tsiafouli MA, Bjornlund L, et al. Soil food web properties explain ecosystem services across European land use systems. Proc. Natl. Acad. Sci. 2013;110:14296–301.

87. Tsiafouli MA, Thébault E, Sgardelis SP, de Ruiter PC, van der Putten WH, Birkhofer K, et al. Intensive agriculture reduces soil biodiversity across Europe. Glob. Chang. Biol. 2015;21:973–85.

88. Frey S., Elliott E., Paustian K. Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. Soil Biol. Biochem. 1999;31:573–85.

89. Bardgett RD, Hobbs PJ, Frostegård Å. Changes in soil fungal:bacterial biomass ratios following reductions in the intensity of management of an upland grassland. Biol. Fertil. Soils. 1996;22:261–4.

90. de Vries FT, Hoffland E, van Eekeren N, Brussaard L, Bloem J. Fungal/bacterial ratios in grasslands with contrasting nitrogen management. Soil Biol. Biochem. 2006;38:2092–103.

91. Wardle DA, Bardgett RD, Klironomos JN, Setälä H, van der Putten WH, Wall DH. Ecological linkages between aboveground and belowground biota. Science. 2004;304:1629–33.

Chapter 2:

Cropping practices manipulate abundance patterns of root and soil microbiome members paving the way to smart farming

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Abstract

Background*:* Harnessing beneficial microbes presents a promising strategy to optimize plant growth and agricultural sustainability. Little is known to which extent and how specifically soil and plant microbiomes can be manipulated through different cropping practices. Here, we investigated soil and wheat root microbial communities in a cropping system experiment consisting of conventional and organic managements, both with different tillage intensities.

Results*:* While microbial richness was marginally affected, we found pronounced cropping effects on community composition, which were specific for the respective microbiomes. Soil bacterial communities were primarily structured by tillage, whereas soil fungal communities responded mainly to management type with additional effects by tillage. In roots, management type was also the driving factor for bacteria but not for fungi, which were generally determined by changes in tillage intensity. To quantify an 'effect size' for microbiota manipulation, we found that about 10% of variation in microbial communities was explained by the tested cropping practices. Cropping sensitive microbes were taxonomically diverse and they responded in guilds of taxa to the specific practices. These microbes also included frequent community members or members co-occurring with many other microbes in the community, suggesting that cropping practices may allow manipulation of influential community members.

Conclusions*:* Understanding the abundance patterns of cropping sensitive microbes presents the basis towards developing microbiota management strategies for *smart* farming. For future targeted microbiota management – e.g., to foster certain microbes with specific agricultural practices – a next step will be to identify the functional traits of the cropping sensitive microbes.

Keywords: soil and root microbiomes, microbial co-occurrence, network analysis, cropping practices, microbiota management, smart farming

Background

Agricultural intensification has resulted in an increased production of staple crops such as wheat, rice, and maize and lead to greater food security for a continuously growing world population [1,2]. Despite these benefits, there is increasing awareness about the adverse environmental impacts arising from the intensive practices of modern agriculture. These include increased greenhouse gas emissions and nutrient leaching as a result of intensive fertilizer application [3], increased soil erosion [4], and detrimental effects on biodiversity [5,6]. To alleviate such deleterious effects, an ecological intensification has been proposed that focuses on meeting standards of environmental quality while promoting and maintaining organisms that provide beneficial ecosystem services [7,8]. A number of practices improve the sustainability of agriculture, including organic farming [9] and reduced or no-tillage [10]. These practices aim to enhance soil fertility while maintaining crop yields through supporting a diverse and active soil biota [11]. Soil biota includes microbes such as bacteria and fungi that collectively function as a microbiome. Bacteria and fungi regulate many ecosystem processes and play key roles in nutrient cycling through decomposition of organic matter, and transformation and fixation of important soil nutrients like nitrogen and phosphorus [12].

Aside from the environmental benefits of organic agriculture [13] and less intensive tillage regimes [10], there is still debate about the effects of these cropping practices on belowground microbial communities. In general, arable management affects community composition and diversity; although such effects may depend on the microbial kingdom being studied and the different farming systems being compared [14,15]. However, there are few agricultural experiments comparing conventional and organic farming practices [16], and fewer that compare different management types and tillage intensities [17]. Therefore, an agricultural experiment combining these two aspects at a single site allows to separate the effects of management type and tillage on microbial communities and minimize variation caused by soil spatial heterogeneity. The Farming System and Tillage experiment (FAST) was established in 2009 near Zürich to address this for the main arable cropping systems in Switzerland (Additional file 1: Fig. S1). These cropping systems are namely, conventional and organic management types, with different tillage intensities (no-tillage, reduced-tillage, and intensive tillage). Wittwer *et al.,* [18] described the design of the FAST experiment and provided agronomic insights into the effects of different farming practices on winter wheat and maize yields. However, the effects of management types and different tillage intensities on belowground microbial communities at the site have not been explored.

Soil microbial communities and their associated functions largely determine the productivity of agroecosystems [19]. The composition of the soil microbiome presents the major driver in shaping the bacterial and fungal communities associated with plant roots [20,21]. The root microbiome is an important determinant for plant growth and health by assisting in nutrient uptake, supporting abiotic stress tolerance, and protecting the host from pathogens [20,22]. A number of recent microbial community surveys have described the root microbiomes of *Arabidopsis thaliana* [23,24], clover [25], maize [26], rice [27], sugarcane [28] and grapevine [29], and reported significant effects of soil type on root microbiome composition. If soil and root microbial communities are closely linked, root microbial communities may also be affected by agronomic practices [30]. To date, the effects of agricultural practices on root microbial communities remain still poorly understood, owing to contrasting reports and the use of low-resolution fingerprinting methods [31]. Using high throughput sequencing, we aimed to unravel how root microbial communities respond to conventional and organic agriculture and various tillage regimes.

Members of the soil and root microbiome interact directly and indirectly with each other, and a tool for better understanding of these potential interactions is co-occurrence network analysis [32,33]. Long used in the social sciences to analyze relationships between humans [34], network analyses have recently been applied in soil microbial ecology to explore patterns of community assembly [35], visualize response patterns of different taxonomic groups to agronomic practices [36], and to identify individual microbiome members that significantly influence community composition [37]. It was recently shown that soils under conventional and organic management harbor distinct microbial networks in each farming system [38]. To date, the effects of different cropping practices on co-occurrence patterns in the root microbiome remain unexplored.

From the perspective of microbiome management, it is important to understand which microbes are sensitive to cropping practices and whether they possess specific network properties. Microbes that frequently co-occur with many others are referred to as keystone taxa because they may play an ecologically important role by determining community dynamics and microbiome functioning [37–39]. It is unclear whether keystone taxa in soil and root microbiomes are responsive to cropping practices. More importantly, are cropping sensitive microbes solitary community members, or do they belong to guilds of simultaneously responding taxa? Are they frequent or not? Such information is relevant for implementing agricultural management strategies to promote specific microbes that contribute to soil fertility and plant health.

With these ideas in mind, we investigated the impact of cropping practices at the FAST experimental site on soil and root bacterial and fungal communities in winter wheat using amplicon sequencing and network analysis. We specifically asked: (1) Do soil and root microbial communities differ in their responses to management type and tillage intensities? (2) Which microbes are the indicator taxa for particular cropping practices (conventional vs. organic; reduced vs. intensive tillage)? (3) How do cropping practices impact co-occurrence patterns of soil and root associated microbes? (4) What are the network characteristics (abundance, degree of co-occurrence, and keystoneness) of cropping sensitive microbes?

Results

Soil and root microbiota

We conducted separate bacterial and fungal community profiling of 16 soil and 16 winter wheat root samples from of the FAST experiment (Additional file 1: Fig. S1) to investigate the effects of management type and tillage intensity on microbial communities. The bacterial community profiling yielded a total of 639,440 high-quality sequences (range: 11,192 - 37,255; median: 18,122; Additional file 2). Fungal profiling yielded 962,619 sequences, ranging between 9,138 and 48,750 sequences per sample (median: 30,284). We identified 2,972 bacterial, 3 archaeal and 1,975 fungal operational taxonomic units (OTUs) across all samples (Additional file 1: Fig. S2).

Plant roots and soil present different microbial habitats with specific sets of microbes (Fig. 1). Taxonomies are described in the Supplementary Results and Fig. S3 (Additional file 1). We visualized and quantified the differences between microbial communities (β-diversity) using unconstrained principal coordinates analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities. Microbial communities of root and soil clearly separated along Axis 1 (Additional file 1: Fig. S4). The discrete outlier in the bacterial communities was consistent with relatively low soil pH in one subplot. We only noted a subtle clustering by cropping practices along Axis 2 where the root fungi tended to group by the intensity of tillage. PERMANOVA confirmed the marked differences between the two microbial habitats (bacteria $R^2 = 0.602$, $P < 0.001$; fungi $R^2 = 0.376$, $P < 0.001$) and smaller but significant impact of cropping practices (bacteria $R^2 = 0.086$, $P < 0.05$; fungi $R^2 = 0.102$, $P < 0.05$; Additional file 1: Table S2).

For α-diversity analyses, we rarified the communities to 11,000 (bacteria) and 9,000 (fungi) sequences per sample, which captured most of the observed OTU richness (Additional

file 1: Fig. S5). Soils supported higher species richness than roots with bacterial communities being greater in richness than fungi (Additional file 1: Fig. S5, Table S3). In both soil and root communities, bacteria and fungi richness was highest in O-IT samples with significant effect for bacterial communities in root samples. To conclude, plant root and soil microbiota differ markedly in richness, composition and taxonomy.

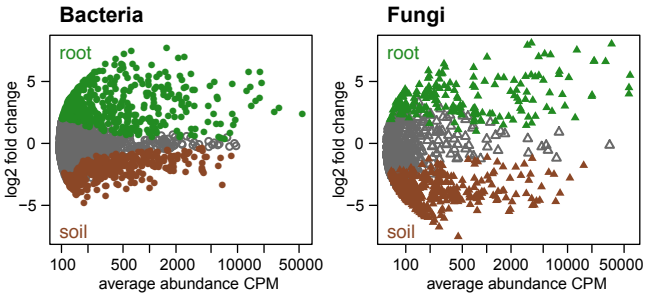

Figure 1: Soil and roots harbor specific sets of microbes. MA plots displaying the abundance patterns of bacteria and fungi in soil and root microbiomes. X-axis reports average OTU abundance (as counts per million, CPM), and Y-axis log2-fold change (root relative to soil). Root and soil-specific OTUs were colored in green and brown, respectively and non-differentially abundant OTUs are in grey (likelihood ratio test, $p<0.05$, FDR corrected).

Cropping system effects on soil and root microbial communities

For in-depth analysis of cropping system effects on root and soil microbial communities, we employed canonical analysis of principal coordinates (CAP). Partial CAP - constrained by cropping system - highlighted a tillage effect on soil bacteria and both management and tillage effects on soil fungal communities (Fig. 2). PERMANOVA confirmed the significant effect of cropping systems on both soil microbial communities (Additional file 1: Table S4). Pairwise tests revealed significant differences between the two conventional and O-RT treatments but not O-IT treatments for soil bacteria. For the soil fungi, significant differences were found between the low-intensity tillage treatments and O-IT but not C-IT treatments.

Different patterns were observed for the root microbiota. Root bacteria formed four distinct clusters in the ordination with axis 1 again separated the samples by management type and axis 2 separated the samples by tillage intensity. Pairwise PERMANOVA comparisons detected significant differences between the two conventional treatments and O-RT but not O-IT samples. For root fungal communities, CAP separated the O-RT samples along Axis 1 and the C-NT samples from the other treatments on Axis 2. PERMANOVA also confirmed a general effect of cropping system but no pairwise differences on community dissimilarity were found.

Since β-diversity can be driven by true biological differences, differences in group dispersion (variance), or both [40], we tested for differences in dispersion for both soil and root microbiota using BETADISP. The lack of significance in these dispersion tests suggested that differences between cropping systems were driven primarily by true biological differences and not an artifact of differences of within-group dispersion (Additional file 1: Table S4). In summary, while tillage-driven differences were seen in the soil bacterial community, the management type appeared to be the main driving factor in root bacteria. Conversely, root fungal communities did not strongly respond to management type induced changes in soil, and instead were determined by changes in the tillage intensity.

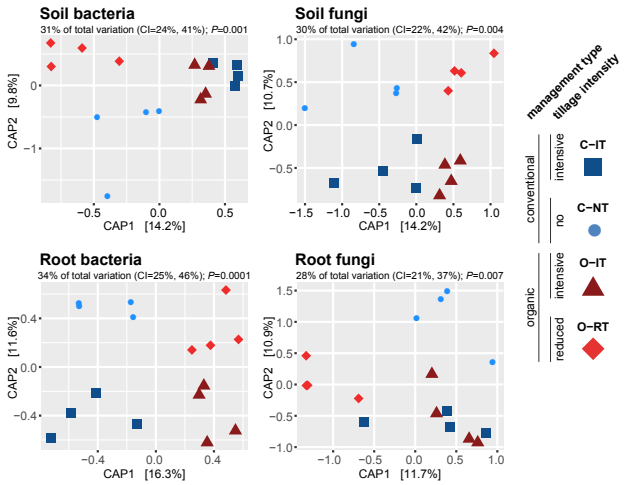

Figure 2: Effects of cropping practices on soil and root bacteria and fungi. Separate CAP ordinations using Bray–Curtis distance were performed for bacteria and fungi, both in roots and soil. CAP analyses were constrained by the factor 'cropping systems', and the explained fraction of the total variance is indicated above the plots (with 95% confidence interval, significance assessed with 10^4 permutations). Percentage of variation given on each axis refers to the explained fraction of total variation.

Identifying cropping sensitive OTUs

We employed indicator species analysis to identify individual bacteria (bOTUs) and fungi (fOTUs) in soil and root communities whose abundances varied between the different cropping systems, and we summarized the analysis with a bipartite network (Fig. 3; Additional file 3). Patterns were reminiscent of the effects seen in the previous diversity analyses. For instance, the high number of soil bacteria OTUs that were shared between the intensive tillage reflects the close clustering of these samples in the ordination. Similarly, consistent with the finding that both management type and tillage intensity explain variation among soil fungi, we found high numbers of indicator OTUs specific to one-cropping system.

As indicator OTUs were solely identified based on correlation, we validated them using likelihood ratio tests implemented in edgeR (41; Additional file 3). Finally, we defined the

OTUs that were supported by both methods as *c*ropping *s*ensitive OTUs (hereafter: *cs*OTUs). In soil, we found a total of 53 and 70 bacteria and fungi *cs*OTUs, respectively (Additional file 1: Fig. S6). As approximation for an 'effect size' of cropping practices on microbial communities, we calculated these bacteria and fungi *cs*OTUs to account for 8.3% and 9.9% of the total soil community sequences. Similarly, we identified 62 and 36 *cs*OTUs for root bacteria and fungi, corresponding to an effects size of 14.2% and 5.0%, respectively. Consistent with the previous conclusion that cropping practices affected soil and root communities differently, we saw little overlap between bacteria and fungi *cs*OTUs comparing root and soil samples. While the identified *cs*OTUs responded to specific cropping systems, they did not exhibit a particular taxonomic pattern with cropping system (Additional file 1: Figs. S7-S12, Supplementary Results). Taken together, each cropping system supports a specialized subset of soil and root bacteria and fungi, while the majority of the communities are shared between management types and tillage regimes.

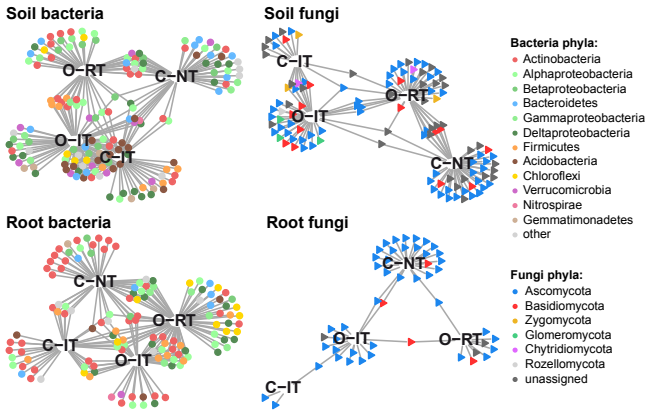

Figure 3: Bipartite networks display cropping system specific OTUs in the soil and root bacterial and fungal communities as determined using indicator species analysis. Circles represent individual bacteria and triangles fungi OTUs that are positively and significantly associated (p<0.05) with one or more of the cropping systems (association(s) given by connecting lines). OTUs are colored according to their Phylum assignment.

Cropping effects on microbial co-occurrence patterns

Lastly, we explored the extent to which management types and tillage regimes impacted co-occurrence patterns in microbial communities. We first constructed separate co-occurrence networks for soil and root bacterial and fungal communities and determined their properties (see the "Methods" section). Consistent with the α-diversity analyses (Additional file 1: Fig. S5), the soil bacteria network comprised the highest number of significantly co-occurring OTUs, followed by intermediate and similar numbers in the soil fungi and root bacteria networks (Additional file 1: Fig. S13). Consequently, network connectivity (measured by average number of connections per OTU) was higher in the soil bacteria and soil fungi

networks than the root bacteria network. The root fungi network comprised the fewest OTUs and was the least complex. We also mapped the *cs*OTUs (as defined in Additional file 1: Fig. S6) into the microbial networks, and we found them agglomerating according to management type and/or tillage intensity (Additional file 1: Fig. S13).

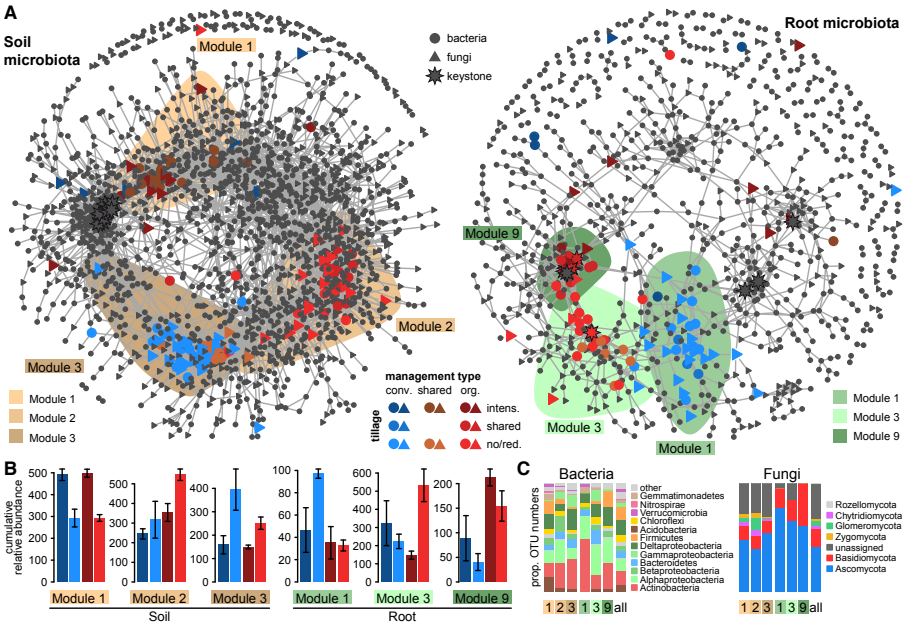

Figure 4: Co-occurrence patterns of cropping sensitive OTUs. **(**A) Co-occurrence networks visualizing significant correlations (ρ>0.7, *p<0.001*; indicated with grey lines) between bacteria and fungi OTUs in soil and root communities. Circles indicate bacteria, triangles fungi, and keystone OTUs are represented with asterisks (Table 1). OTUs are colored by their association to the different cropping systems (as defined in Additional file 1: Fig. S6; grey OTUs are insensitive to cropping practices). Shaded areas represent the network modules containing *cs*OTUs as defined in Additional file 1: Fig. S14. (B) Cumulative relative abundance (as counts per million, CPM; y-axis in 1000x) of all bacteria and fungi of the cropping sensitive modules in soil and root networks. The cumulative relative abundance in samples of C-IT (dark blue), C-NT (light blue), O-IT (dark red), O-RT (light red) cropping systems indicates the overall response of cropping sensitive modules to the different farming practices. (C) Qualitative taxonomic composition of cropping sensitive modules is reported as proportional OTUs numbers per Class (Bacteria) and Phylum (Fungi) and compared to the overall taxonomic distribution in the entire dataset (column "all").

Next, we explored the distribution patterns of *cs*OTUs in meta co-occurrence patterns of bacteria and fungi in soil and root communities (Fig. 4a, Table 1). We found that the abundance patterns of inter-kingdom microbial associations also responded to cropping practices. We noted in the soil and root meta-networks that three modules contained relatively high proportions of *cs*OTUs (Additional file 1: Fig. S14; Additional file 5). The type of sensitivity of these module members to the specific cropping systems (Fig. 4b) and their distribution in the network partially reflected the drivers of community dissimilarity seen in the CAP ordinations (Fig. 2). For example, the effect of tillage intensity in the soil communities was apparent with a discrete module (M1) in the soil network, containing *cs*OTUs specific to

intensive tillage practices. M1 was separated from two other modules (M2 and M3) that primarily contained *cs*OTUs specific to the O-RT and C-NT cropping systems (Fig. 4a,b). Similarly, management type presented the main driver in root communities (Fig. 2), and the numerous *cs*OTUs assigned to organic management were predominantly located in modules M3 and M9 and separated from module M1 containing primarily conventional management specific OTUs (Fig. 4b). Furthermore, the separation of the two modules containing *cs*OTUs specific to organic production systems appeared to reflect differences in tillage practices (Fig. 4a,b). All the management and tillage responsive modules in soil and roots comprised a taxonomically broad set of bacteria and fungi (Fig. 4c), revealing that the different cropping practices do not target specific microbial lineages.

Table 1: Properties of soil and root meta co-occurrence networks

Community	[1]OTUs		[2]Connections			[3]Connectivity	[4]Keystone		[5]*cs*OTUs	
	Bacteria	Fungi	Bac-Bac	Fun-Fun	Bac-Fun	Network wide	Bacteria	Fungi	Bacteria	Fungi
Soil	1197	747	1904	1111	2270	5.4	10	9	51 (0)	69 (0)
Root	688	239	855	159	434	3.1	9	0	57 (5)	33 (0)

[1] Number of network nodes
[2] Number of network edges
[3] Mean number of connections per node
[4] Number of Keystone OTUs
[5] Number of cropping sensitive OTUs present in the network (number of keystone OTUs therein)

The *cs*OTUs were identified among low count as well as among highly abundant soil and root taxa (Fig. 5). In soil, they had low to medium degrees of co-occurrence, while in roots they were also found among OTUs that co-occurred with many other taxa. In roots, we observed that 'organic' *cs*OTUs exhibited higher degrees of co-occurrence than 'conventional' *cs*OTUs. With the exception of five root bacteria OTUs, the majority of keystone OTUs was not sensitive to cropping practices (Table 1, Additional file 1: Table S5). The keystone *cs*OTUs were from the Firmicutes (*bOTU23*, *bOTU119* family *Peptostreptococcaceae*, *bOTU36* family *Erysipelotrichaceae*), the Chloroflexi (*bOTU949*, family *Chloroflexaceae*) and the Actinobacteria (*bOTU530* family *Microbacteriaceae*) and had higher abundances in roots from organically managed plots (Additional file 1: Fig. S11).

Taken together, we show that the differing cropping practices markedly alter co-occurrence patterns of numerous bacteria and fungi, and these impacts affected microbiome members largely independent of their abundance and connectivity.

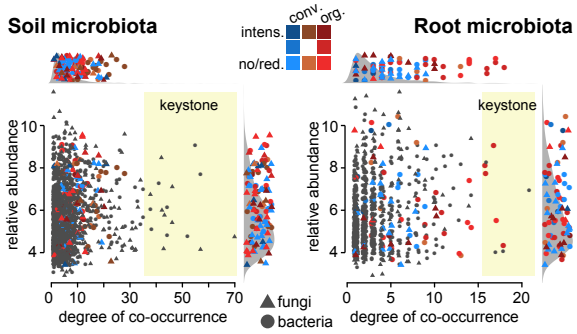

Figure 5: Degree of co-occurrence and abundance of *cs*OTUs. Relative abundance (as counts per million, CPM) of all OTUs from the soil and root microbiome co-occurrence networks (Fig. 4) was plotted as a function of their degree of co-occurrence. Circles and triangles refer to bacteria and fungi, respectively. OTUs were colored by their association to the different cropping systems and keystone OTUs (top 1% degree of co-occurrence) have yellow background. Side panels recapitulate the distributions of co-occurrence degrees and abundance for the *cs*OTUs (shapes colored by association to cropping systems) compared to the density of all.

Discussion

While considering the effects of agricultural practices on soil microbial communities, previous studies have often been limited to the examination of single factors like management type [42–44], tillage [45–47], soil amendments [48–50], or focused on either the bacterial or fungal kingdom. Hartmann *et al.,* [36] investigated effects of management type on soil bacteria and fungi in a multi-factor long-term agricultural experiment. Our motivation was to expand on these efforts by examining cropping system effects on *root* microbes and by also including the effect of tillage into the experimental design. To our knowledge, this is the first study to investigate how conventional and organic managements, both with different tillage regimes, influence bacterial and fungal communities in both soil and root compartments. The strength of the FAST experiment is that management and tillage effects can be studied factorially and independent of possibly confounding variables such as soil types. However, a broader generalization of the presented findings requires further studies with greater sample numbers, from multiple sites, across different climatic conditions and accounting for temporal and special variation in root and soil microbiomes.

Differential responses of soil and root microbiomes

The specific sets of microbes in roots and soil (Fig. 1) explain the clear separation by compartment in the ordination analyses (Additional file 1: Fig. S4). This distinctiveness of the plant root microbiome was also found in previous studies of other plant species [23,24,25,26,28,29,51]. Plants recruit a root microbiome in their early life stages from a larger pool of soil microbes [27,52]. The initial composition of this soil microbial pool is the most influential factor determining the composition of root microbial communities [23,24,53,54].

Therefore, we also investigated if root bacterial and fungal communities reflected cropping system driven differences in soil microbial communities (see Additional file 1: Supplementary Discussion).

We observed compartment-specific responses of bacteria and fungi to the tested cropping practices (Fig. 2, Additional file 1: Table S4). Dissimilarities in soil bacteria were generally driven by differences in tillage regimes, whereas management type, together with tillage, was influential for the soil fungi. Notably, the most influential cropping factors driving differences in soil communities were not necessarily the most influential in root communities. In the root microbiome, we found that the management type was the most influential factor for the root bacteria, while tillage intensity explained most of the variation in the root fungi. Taken together, our results demonstrate that agricultural management affects soil and root microbial communities differently.

We hypothesize that a combination of timing and nutrient characteristics of root and soil compartments could explain the differential responses of soil and root microbiomes to the cropping practices. By timing, we refer to the different time points between the effective cropping practices (mostly before seeding) and the harvest or sampling of the crop. We assume that the soil microbiome would exhibit the most pronounced differences in response to tillage or manure fertilization shortly after application and that such effects would gradually decline over the length of the growing season until a soil-type specific equilibrium is reached again after disturbance. For the root microbiome, however, crops are sown soon after tillage or manure application and the roots recruit microbes from the most divergent conditions so that the pronounced differences between cropping practices may be ‘fixed’ for a longer time. Hence, primary colonizers that coin the root microbiome assembly at early stages would explain the preservation of precedent management differences. In addition, the nutritional characteristics of the root compartment may contribute to preserving specific management differences. Compared to an oligotrophic soil environment, we consider plant roots a copiotrophic compartment due to the continuous secretion of root exudates. Nutrient-rich organic fertilizers mainly contain copiotrophic gut bacteria from cattle that may also find favorable conditions in the root compartment. We see support for this idea as there is a marked impact of organic management on the root bacteria and not on soil (Figs. 2 and 3) and because the only bacteria with high degrees of co-occurrence were exclusively found in the root microbiome networks and were specific to organic farming (Fig. 5). Future experiments are needed to test these hypotheses. Such experiments would include e.g., the quantification of cropping induced microbiome differences of soil and root samples in time series throughout

the growing season or manure application tests that uncouple nutritional from microbial components (e.g., applications of nutrient-free microbial extracts from slurry or extracts with inactivated slurry microbiota).

We confirmed the soil to be more diverse than the root microbial communities [20,55], however, we only found marginal impacts of the cropping systems on bacteria and fungi α-diversity (Additional file 1: Fig. S5, Table S3, see Supplementary Discussion). Hence, the different cropping practices affected species richness to smaller degree than community composition. This is consistent with previous observations that species richness was less variable in their responses to environmental factors (i.e., different cropping systems) than species composition [56,57]. Changes in microbial community composition may not necessarily lead to altered diversity or richness because changes of some taxonomic groups may be compensated by changes in others [57], and because univariate measures of diversity and richness mask relationships between individual and groups of taxa [58].

Cropping sensitive microbes

We identified *c*ropping *s*ensitive *cs*OTUs in both soil and root microbial communities (Additional file 1: Fig. S6), and they function as indicator taxa to explain the β-diversity patterns by cropping practices (Fig. 2). For example, the higher relative abundance of bacteria *cs*OTUs from the Firmicutes in organically managed plots (Additional file 1: Fig. S7) was congruent with a separation by management type in CAP analysis of soil and root communities. The association of Firmicutes OTUs to organic plots that receive manure fertilizer was found earlier [36]. In our case, we noted that OTUs representing four families within the Firmicutes, *Peptostreptococcaceae* (*bOTU23, bOTU119,* genus not assigned)*, Clostridiaceae* (*bOTU341,* genus *Clostridium*), *Erysipelotrichaceae* (*bOTU36,* genus *Turicibacter*), and *Lachnospiraceae* (*bOTU1403,* genus *Butyrivibrio*) had higher abundances in soil and root samples from organically managed plots (Additional file 1: Figs. S9, S11). It is possible that the higher abundance of these OTUs is a direct result of manure application, as bacteria from these families have previously been isolated from cattle manure [59] or reported in such community surveys [60] and are also common in waste products of other livestock [61].

Although, inference of ecological function from OTU data must be interpreted cautiously, we inspected the *cs*OTUs for taxa with known functions of potential importance in agriculture. Notably in soil fungi, we found two OTUs from the genus *Gibberella* (*fOTU57*, *fOTU32,* family *Nectriaceae*) that were responsive to tillage intensities and had higher abundances in no and reduced tillage samples (Additional file 1: Fig. S10). *Gibberella*,

specifically *Gibberella zeae* (*fOTU57*), is a teleomorph of *Fusarium graminearum*. This pathogen of wheat causes *Fusarium* head blight disease, which is responsible for wheat yield losses worldwide [62]. Similarly, in root fungi, we noted an *Alternaria* OTU (*fOTU63* family *Pleosporaceae*) with a higher abundance in C-NT samples (Additional file 1: Fig. S12). Species of this genus are also known pathogens of wheat and cause leaf blight disease [63]. These examples could suggest that less intensive tillage systems, may favor potentially pathogenic taxa. In a study examining the functional role of plant-beneficial Pseudomonads and soil suppressiveness at the FAST experiment, the soil from the O-RT plots tended to be more suppressive to the soil-borne pathogen *Pythium ultimum* to than the soil from C-NT plots (*personal communication,* Dr. M. Maurhofer, ETH Zurich). It is generally difficult to infer ecological function of a microbe solely based upon a taxonomy assignment [64] Thus, hypotheses about microbial functions of *cs*OTUs need to be tested using other methods such as (meta-)genome or (meta-)transcriptome sequencing or by functional assays with isolated strains to experimentally test how the cropping sensitive microbes affect plant performance [65].

Cropping system effects on microbial co-occurrence

In both soil and root meta-networks, we identified modules containing high proportions of OTUs responding similarly to different cropping practices (Fig. 4, Additional file 1: Fig. S14). We observed that *cs*OTUs grouped in distinct modules that reflected the different cropping systems. We concluded that larger groups of microbes responded in a similar manner to the specific cropping practices and therefore, clustered together in the soil and root microbial networks. The soil *cs*OTUs exhibited low to medium degrees of co-occurrence in the soil network (Fig. 5), revealing that cropping practices did not affect the highly co-occurring soil microbes, which possibly belong to 'core microbiome' members [66]. This observation suggests that only the 'accessory soil microbiome' could be manipulated through cropping practices. In contrast, the *cs*OTUs in the root microbiome – in particular the ones that were sensitive to organic farming – included members with high degrees of co-occurrence (see keystones below). This possibly means that influential community members can also be manipulated with organic cropping practices in the root microbiome. We see additional support for this hypothesis in the observation that *cs*OTUs also included abundant microbiome members.

Keystone taxa are thought to frequently interact with many other taxa, thereby playing an important role in the overall community [67,68]. We found the effects of cropping system

were mostly limited to non-keystone taxa despite significant effects of cropping system on β-diversity and network patterns (Figs. 2 and 4). Nevertheless, we found five keystone OTUs to be cropping sensitive in the root bacteria (Additional file 1: Table S5). Three of these – *bOTU23* and *bOTU119* (both *Peptostreptococcaceae*) and *bOTU36* (*Erysipelotrichaceae*) – are common bacteria in cattle manure or livestock waste samples [59,60,61], and they had higher abundances in organically managed plots (Additional file 1: Fig. S11). This finding suggests the hypothesis that manure application to soil may introduce taxa to the root microbiome with keystone function. Hence, the possible introduction of microbes from manure and their particular influence on root microbiome functioning presents a high research priority.

It is important to stress that co-occurrence networks visualize correlative relationships between taxa that include true ecological interactions (e.g., mutualism), but also non-random processes (e.g., niche-overlap), and therefore, do not necessarily reflect direct interactions between taxa [33,69]. Future experiments will assess whether the identified keystone or cropping sensitive species directly influence other members of the microbiome or indirectly influence host performance and fitness, thereby affecting other community members [37]. Nevertheless, co-occurrence networks are a useful tool for exploring abundance patterns in complex microbial communities and could be useful in designing future experiments. For example, in combination with reference stocks of microbial isolates, plant growth experiments can be conducted in which the presence/absence or relative abundance of keystone taxa identified by network analysis can be manipulated and the effects on plant growth and development can be scored [65].

Conclusions

The concept of ‘smart farming’ postulates the use of state-of-the-art (originally sensing) technology to improve the quality, quantity, and sustainability of agricultural production [70]. Its central promises are targeted and site-specific interventions with ‘intelligent’ agricultural management. Here, we propose that agricultural microbiota manipulations and management strategies shall also be considered as ‘smart farming’. The goal is to integrate beneficial plant microbiome traits (e.g., those improving plant growth, nutrient use efficiency, abiotic stress tolerance, and disease resistance) into sustainable agricultural production [71].

As a basis for implementing microbiota management strategies into *smart* cropping systems, we showed here to which extent and how the different cropping practices permit the manipulation of soil and root microbiota. The types of land management and tillage intensities had marked influence on dominant or well-connected bacteria and fungi in both soil and roots.

Follow-up studies now need to identify the beneficial traits of cropping sensitive microbes in order to define the microbiome functions that can be manipulated through cropping practices.

Methods

The FAST experiment

All samples in this study were collected from the Farming Systems and Tillage (FAST) experiment near Zürich, Switzerland (47°26'20" N 8°31'40" E). For a detailed description of the FAST experiment see Wittwer *et al.,* [18]. Briefly, the FAST experiment comprises two replicates established beside each other on the same field. The first replicate started in summer 2009 (FAST I) and the second in summer 2010 (FAST II), following a staggered start design. The FAST experiment was designed to compare conventional (C) and organic (O) managements coupled with different tillage regimes. The FAST experiment compares the 4 main cropping systems C-IT, C-NT, O-IT, O-RT. Conventional plots receive synthetic mineral fertilizers, post-emergence herbicides and pesticides and are subjected to either intensive tillage (IT) or no-tillage (NT, with additional use of glyphosate). The corresponding cropping systems are referred to as conventional with intensive tillage (C-IT) or conventional without tillage (C-NT). Organically managed plots are fertilized with cattle slurry, did not receive synthetic herbicides or pesticides, and are subjected to either intensive tillage (IT) or reduced tillage (RT). The cropping systems are referred to as organic with intensive tillage (O-IT) and organic with reduced tillage (O-RT). A full-factorial design would formally require an 'O-NT' treatment instead of an 'O-RT' treatment. While scientifically sound, a no-till regime under organic management is not agronomically practical, because of insufficient weed control without reduced tillage. Additionally, the FAST experiments comprises four cover crop treatments that are applied at the subplot level; however, for this study we only collected root and soil samples from the cover crop treatment planted with a legume species (e.g., Vicia sp.).

Sample collection and DNA extraction

Soil and root samples from *Triticum aestivum* were collected at flowering stage in June 2014 from the second experimental replication (FAST II; Additional file 1: Fig. S1). The FAST experiment was cropped with the same winter wheat variety (cv. Titlis) but differed in seed coating between organic (untreated) and conventional (against seed-borne pathogens) systems (Details: [18]). In total, 32 samples were collected (4 cropping systems (C-IT, C-NT, O-IT, O-RT) * 4 replicates * 2 sample types (soil and root)). Five soil cores (at 10-20 cm depth) were collected in each plot between wheat rows, pooled and immediately frozen at -80ºC until DNA

extraction. Additional bulk soil was collected for chemical analysis (see Additional file 1: Supplementary Methods). In each sampled subplot, whole root systems corresponding to a rooting depth of ~10 cm were collected from five plants and pooled. The roots were rinsed with tap water to remove soil debris, dried by blotting with sterile paper, and stored at -80ºC until DNA extraction. Our sampling method does not discriminate between microbes inhabiting the inner root tissue and the root surface and for simplicity; we refer to these combined habitats of root-associated microbes as 'root' samples.

The entire root systems were first lyophilized for 48 hours and then ground to a fine powder in a ball mill. DNA was extracted from a 300 mg soil or root (dry weight) subsample using the NucleoSpin Soil DNA extraction kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions, except each sample was extracted twice and the supernatants pooled to maximize DNA yield. Extracted DNA was quantified using a Quant-iT Picogreen dsDNA Assay Kit (Invitrogen, Eugene, OR USA) on a Varian Cary Eclipse fluorescence spectrometer (Agilent Technologies, Santa Clara, CA USA).

PCR, library preparation and sequencing

The 16S rRNA gene amplicon library was generated using the PCR primers 799F [72] and 1193R [73]. The ITS amplicon library was generated using the PCR primers fITS7 [74] and ITS4 [75]. The primers were extended at the 5`end with an error-tolerant barcode for multiplexed library sequencing (Additional file 2). We refer to Additional file 1: Supplementary Methods for details in PCR setup, cycling conditions (Additional file 1: Table S1) and the protocol for library preparation. The libraries were sequenced on the MiSeq Instrument (Illumina, San Diego, USA) using a 600 cycle v3 Sequencing kit, paired-end 2x 300 cycle sequencing mode at the Functional Genomics Center Zurich (www.fgcz.ch).

Bioinformatics

Raw reads were processed using a custom-developed bioinformatics pipeline whose command-line based script is provided as Additional file 4. Reads were pre-quality filtered and trimmed at the 3'-end to 280 bp using PRINSEQ [76] and then merged with FLASH [77]. Sequences were de-multiplexed using Cutadapt [78] and were quality-filtered with PRINSEQ. For operational taxonomic unit (OTU) delineation the 16S rRNA gene sequences were trimmed to the fixed length of 360 bp, sorted by abundance, de-replicated, and clustered to OTUs (≥97%, singletons removed) with UPARSE [79]. Chimeric sequences were screened using UCHIME [80] against the GOLD database [81] and removed. Taxonomy assignment was performed using the SILVA database (v119; [82] with the RDP classifier as implemented in

QIIME [83]. ITS sequences were processed similarly, except they were trimmed to 220 bp and chimeric sequences were screened against the UNITE database [84]. Taxonomy was assigned using the UNITE database (v7.0) with the RDP classifier in QIIME.

Data analysis in R

All statistical analyses were conducted in R v3.3.0 [85]. The R script and all necessary input files are provided as Additional file 5. Additionally, a workflow of the data analysis steps presented below and the figures generated from each step is given in Figure S2 (Additional file 1). Briefly, the bacteria OTUs (bOTUs) and taxonomy tables were filtered to exclude OTUs classified as chloroplasts and mitochondria. Similarly, fungi OTUs (fOTUs) classified as plant, protist, or whose kingdom or phylum was unassigned were removed.

Alpha diversity: Rarefaction analysis was performed in QIIME on the filtered OTU tables that were exported from R. The OTU tables were rarefied from 1,000 to 37,000 (bacteria) or 1,000 to 48,000 (fungi) sequences per sample with a step size of 1,000 and 100 iterations at each step. Estimates of α-diversity (observed OTU richness) were calculated at each rarefaction level in QIIME (Additional file 1: Fig. S3a). We tested the effects of sample type and cropping system on observed species richness for each kingdom individually. For this, we randomly selected one file containing α-diversity estimations at 11,000 (bacteria) and 9,000 (fungi) sequences per sample from QIIME. We tested for differences between soil and root sample using a Student's t-test. We then assessed the effects of experimental block and cropping system on observed species richness using 2-way ANOVA within each kingdom and sample type separately. Because cropping system was confounded within experimental block, we did not test for the *Block*Cropping System* interaction. Significant differences between cropping systems were assessed using Tukey's Honest Significant Differences test using the R package TukeyC [86].

Beta diversity: We conducted a general analysis of β-diversity on the bacterial and fungal communities comparing soil and root samples together (Additional file 1: Fig. S3a) and then subsequently we performed more specific hypothesis testing on the soil and root communities individually (Additional file 1: Fig. S3b). For the general analysis, we normalized the filtered OTU sequence counts for each microbial kingdom separately using the 'trimmed means of M' (TMM) method with the BioConductor package *edgeR* (10) and expressed the normalized counts as relative abundance counts per million (CPM). We then performed unconstrained principle coordinates analysis (PCoA) on Bray-Curtis dissimilarities to quantify the major variance components of β-diversity in each kingdom. Ordination analyses were

performed using the R package *phyloseq* [87]. We tested for sample type and cropping system effects on community dissimilarity with permutational analysis of variance (PERMANOVA) using the functions *adonis* in the *vegan* package with 10^4 permutations [88].

For the separate in-depth analyses of each microbial kingdom and for each sample type (soil and root), we additionally applied the following sequence count threshold to the OTU tables: we selected OTUs with at least two sequences (avoiding single-count OTUs) in at least four samples (the number of replicates per treatment). We considered OTUs remaining after this thresholding step to be the soil and root communities. We normalized the communities using the TMM method and expressed the values as relative abundance CPM. We then performed multivariate analysis of microbial diversity based on the steps outlined by Anderson and Willis [89]. This included: a constrained analysis of principal coordinates (CAP) testing the effect of the cropping systems, statistical testing of the cropping system hypothesis, and identification of the OTUs responsible for the observed effects (see below). All ordination analyses were performed using the R package *phyloseq* [87]. Statistical significance of the CAP was assessed using the *permutest* function in the *vegan* package [88] with 10^4 permutations. We tested for cropping system effects on community dissimilarity with permutational analysis of variance (PERMANOVA) and permutational analysis of multivariate dispersions (BETADISP) using the functions *adonis* and *betadisp*, respectively, in the *vegan* package with 10^4 permutations. Where applicable, pairwise differences between the cropping systems were assessed with the function *pairwise.perm.manova* from the package *RVAideMemoire* [90].

Identification of cropping sensitive OTUs (csOTUs): We employed complementary approaches to identify the OTUs responsible for the observed effects. We used correlation based indicator species analysis with the R package *indicspecies* [91] to calculate the point-biserial correlation coefficient *(r)* of an OTU's positive association to one or a combination of cropping systems. The analysis was conducted with 10^4 permutations and considered significant at $p<0.05$. Additionally, we tested for differential OTU abundance between one or more of the cropping systems of soil and root communities (same thresholded OTU tables) of both kingdoms using likelihood ratio tests (LRT) with the R package *edgeR* [41]. OTUs whose abundances were identified as differing between one or more of the cropping systems at a false discovery rate (FDR) corrected value of $p<0.05$ were considered to be cropping system responsive. We then defined OTUs that were confirmed by both indicator species analysis *and* LRT as *c*ropping *s*ensitive OTUs (*cs*OTUs).

Bipartite networks: We visualized the significant ($p<0.05$) OTU associations to one or more of the different cropping system from the indicator species analysis using bipartite

networks. The networks were constructed using the Fruchterman-Reingold layout with 10^4 permutations as implemented in the R package *igraph* [92].

Co-occurrence networks: We constructed two types of co-occurrence networks. For all networks, we utilized the TMM normalized CPM counts and conducted Spearman rank correlations between OTUs and visualized the positive, significant correlations (ρ>0.7 and p<0.001). All networks were visualized with the Fruchterman-Reingold layout with 10^4 permutations in *igraph*.

For the in-depth assessment of soil and root bacterial and fungal communities, we performed Spearman rank correlations between all pairs of bacteria and all pairs of fungi OTUs within the soil and root communities separately. We calculated the descriptive and topological network properties with *igraph.* These included: the total number of network nodes (representing OTUs), total number of edges (connections between nodes representing positive, significant correlations between OTUs), and degrees of co-occurrence (number of direct correlations to a node).

We then constructed meta-networks to visualize correlations between bacteria and fungi in the soil and root communities. For this, we combined the TMM normalized CPM counts of bacteria and fungi into separate OTU tables for the soil and root communities. We performed Spearman rank correlations between all pairs of bOTUs and fOTUs. We calculated the network properties mentioned above, and additionally, to explore community structure within the soil and root meta-networks, we identified network modules. These are substructures of nodes with a higher density of edges within groups than between them. For this we utilized the *greedy optimization of modularity* algorithm [93] as implemented in *igraph*.

Microbial taxa that frequently co-occur with other taxa in microbial co-occurrence networks are thought to be ecologically important and potentially play a key role within the microbiome [37,38]. We identified keystone OTUs separately for the soil and root meta-networks and defined them as those nodes within the top 1% of node degree values of each network. We prioritized this simple definition over a more complex method (e.g., based on high degree and low betweenness centrality) because both definitions uncovered largely the same sets of keystone OTUs (data not shown).

Additional Files

Additional file 1: A PDF containing supplementary methods, results, discussion, references, figures and tables. The SUPPLEMENTARY METHODS contain the details about the chemical soil analysis, PCR setup, library preparation and sequencing. The SUPPLEMENTARY RESULTS comprise the global taxonomic profiles of soil and root bacterial and fungal communities and the taxonomic patterns of csOTUs. We discuss the cropping system effects on soil microbial communities and on microbial α-diversity in the SUPPLEMENTARY DISCUSSION. SUPPLEMENTARY FIGURES: **Figure S1** - Experimental layout of the FAST experiment. **Figure S2** - Graphical overview of data analysis. **Figure S3** - Taxonomic profiles at phylum level. **Figure S4** - Unconstrained PCoA ordinations. **Figure S5** - Rarefaction curves. **Figure S6** - Defining cropping sensitive bacteria and fungi in soil and root samples. **Figure S7** and **Figure S8** - Mean relative abundances of cropping sensitive OTUs at phylum and OTU level, respectively. **Figure S9** - Abundant cropping sensitive bacteria bOTUs in soil. **Figure S10** - Abundant cropping sensitive fungi fOTUs in soil. **Figure S11** - Abundant cropping sensitive bacteria bOTUs in roots. **Figure S12** - Abundant cropping sensitive fungi fOTUs in roots. **Figure S13** - Separate co-occurrence networks of bacteria and fungi in soil and root samples. **Figure S14** - Defining modules in root and soil networks. SUPPLEMENTARY TABLES: **Table S1** - PCR cycling conditions. **Table S2** - PERMANOVA results for testing the effects of *Block*, *Sample type* and *Cropping System*. **Table S3** - Statistic results testing for differences in α-diversity. **Table S4** - PERMANOVA results testing the effects of *Block* and *Cropping System* on bacterial and fungal communities in soil and root samples. **Table S5** - Characteristics of keystone OTUs.

Additional file 2: An XLSX table contains the experimental design (Sample ID, sample type and cropping system), chemical soil data and sequencing information (barcodes and sequence counts).

Additional file 3: An XLSX table reporting the indicator species and edgeR results and the assignments to cropping sensitive OTUs and network modules. This information is provided in separate sheets for the bacteria and fungi in soil and roots.

Additional file 4: A zip archive comprising the command line code and necessary input files needed to replicate bioinformatic analysis.

Additional file 5: A zip archive with the R script and necessary input files needed to reproduce all statistical analyses and graphics.

Declarations

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Availability of data and materials:
The raw sequencing data is available from the European Nucleotide Archive. The 16S rRNA gene library was run together with data presented in Hartman *et al.,* [25] and is available under the study accession PRJEB15152 (Sample: SAMEA54297418). The MiSeq run containing the ITS reads is available under the study accession PRJEB21595. We provide the command-line script for the bioinformatic sequence processing as Additional file 4. Similarly, we provide the R script for data analysis and all necessary input files as Additional file 5.

Authors' contributions:
KH, MVDH, and KS conceived the study, participated in its design, and wrote the manuscript. RAW maintains the Farming System and Tillage field experiment and helped with sampling. KH, SB, and KS conducted the experiments and analyzed the data. JCW performed the bioinformatic analysis. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

References

1. Tilman D. Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices. Proc. Natl. Acad. Sci. U. S. A. 1999;96:5995–6000.

2. Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, et al. The Challenge of Food Security. Science. 2012;327:812–8.

3. Matson P, Parton W, Power A, Swift M. Agricultural Intensification and Ecosystem Properties. Science. 1997;277:504–9.

4. Pimentel D, Harvey C, Resosudarmo P, Sinclair K, Kurz D, McNair M, et al. Environmental and economic costs of soil erosion and conservation benefits. Science. 1995;267:1117–23.

5. Vitousek PM, Mooney HA, Lubchenco J, Melillo JM. Human Domination of Earth's Ecosystems. Science. 1997;277:494–9.

6. Stoate C, Boatman ND, Borralho RJ, Carvalho CR, de Snoo GR, Eden P. Ecological impacts of arable intensification in Europe. J. Environ. Manage. 2001;63:337–65.

7. Bommarco R, Kleijn D, Potts SG. Ecological intensification: Harnessing ecosystem services for food security. Trends Ecol. Evol. 2013;28:230–8.

8. Cassman KG. Ecological intensification of cereal production systems: yield potential, soil quality, and precision agriculture. Proc. Natl. Acad. Sci. U. S. A. 1999;96:5952–9.

9. Reganold JP, Wachter JM. Organic agriculture in the twenty-first century. Nat. Plants. 2016;2:15221.

10. Hobbs PR, Sayre K, Gupta R. The role of conservation agriculture in sustainable agriculture. Philos. Trans. R. Soc. London B Biol. Sci. 2008;363:543–55.

11. Mäder P, Fliessbach A, Dubois D, Gunst L, Fried P, Niggli U. Soil fertility and biodiversity in organic farming. Science. 2002;296:1694–7.

12. Bender SF, Wagg C, van der Heijden MGA. An Underground Revolution: Biodiversity and Soil Ecological Engineering for Agricultural Sustainability. Trends Ecol. Evol. 2016;31:440–52.

13. Gomiero T, Pimentel D, Paoletti MG. Environmental Impact of Different Agricultural Management Practices: Conventional vs. Organic Agriculture. Crit. Rev. Plant Sci. 2011;30:95–124.

14. Bünemann EK, Schwenke GD, Zwieten L Van. Impact of agricultural inputs on soil organisms—a review. Aust. J. Soil Res. 2006;44:379.

15. Postma-Blaauw M, Goede R de, Ecology S, February N, Jack H. Soil biota community structure and abundance under agricultural intensification and extensification. Ecology. 2010;91:460–73.

16. Esperschütz J, Gattinger A, Mäder P, Schloter M, Fließbach A. Response of soil microbial biomass and community structures to conventional and organic farming systems under identical crop rotations. FEMS Microbiol. Ecol. 2007;61:26–37.

17. Treonis AM, Austin EE, Buyer JS, Maul JE, Spicer L, Zasada IA. Effects of organic amendment and tillage on soil microorganisms and microfauna. Appl. Soil Ecol. 2010;46:103–10.

18. Wittwer RA, Dorn B, Jossi W, van der Heijden MGA. Cover crops support ecological intensification of arable cropping systems. Sci. Rep. 2017;7:10.1038/srep41911.

19. van der Heijden MGA, Bardgett RD, Van Straalen NM. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol. Lett. 2008;11:296–310.

20. Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 2013;64:807–38.

21. Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufrense A. The importance of the microbiome of the plant holobiont. New Phytol. 2015;206:1196-206.

22. Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and plant health. Trends Plant Sci. 2012;17:478–86.

23. Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, et al. Defining the core Arabidopsis thaliana root microbiome. Nature. 2012;488:86–90.

24. Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, et al. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature. 2012;488:91–5.

25. Hartman K, van der Heijden MG, Roussely-Provent V, Walser J-C, Schlaeppi K. Deciphering composition and function of the root microbiome of a legume plant. Microbiome. 2017;5:10.1186/s40168-016-0220-z.

26. Peiffer JA, Spor A, Koren O, Jin Z, Tringe SG, Dangl JL, et al. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. U. S. A. 2013;110:6548–53.

27. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, et al. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc. Natl. Acad. Sci. 2015;112:E911–20.

28. Yeoh YK, Paungfoo-Lonhienne C, Dennis PG, Robinson N, Ragan MA, Schmidt S, et al. The core root microbiome of sugarcanes cultivated under varying nitrogen fertiliser application. Environ. Microbiol. 2015;18:1338–51.

29. Zarraonaindia I, Owens SM, Weisenhorn P, West K, Hampton-Marcell J, Lax S, et al. The soil microbiome influences grapevine-associated microbiota. MBio. 2015;6:e02527-14.

30. Seghers D, Wittebolle L, Top EM, Verstraete W, Siciliano S. Impact of agricultural practices on the Zea mays L. endophytic community. Appl. Environ. Microbiol. 2004;70:1475–82.

31. Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G. Microbial diversity and soil functions. Eur. J. Soil Sci. 2003;54:655–70.

32. Fuhrman JA. Microbial community structure and its functional implications. Nature. 2009;459:193–9.

33. Faust K, Raes J. Microbial interactions: from networks to models. Nat. Rev. Microbiol. 2012;10:538–50.

34. Newman MEJ. The structure and function of complex networks. SIAM Rev. 2003;45:167–256.

35. Barberan A, Bates ST, Casamayor EO, Fierer N. Using network analysis to explore co-occurrence patterns in soil microbial communities. ISME J. 2012;6:343–51.

36. Hartmann M, Frey B, Mayer J, Mäder P, Widmer F. Distinct soil microbial diversity under long-term organic and conventional farming. ISME J. 2014;9:1177–1194.

37. Agler MT, Ruhe J, Kroll S, Morhenn C, Kim ST, Weigel D, et al. Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. PLoS Biol. 2016;14:e1002352.

38. van der Heijden MGA, Hartmann M. Networking in the Plant Microbiome. PLOS Biol. 2016;14:e1002378.

39. Banerjee S, Kirkby CA, Schmutter D, Bissett A, Kirkegaard JA, Richardson AE. Network analysis reveals functional redundancy and keystone taxa amongst bacterial and fungal communities during organic matter decomposition in an arable soil. Soil Biol. Biochem. 2016;97:188–98.

40. Anderson MJ. A new method for non parametric multivariate analysis of variance. Austral Ecol. 2001;26:32–46.

41. Robinson MD, McCarthy DJ, Smyth GK. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26:139–40.

42. Sugiyama A, Vivanco JM, Jayanty SS, Manter DK. Pyrosequencing Assessment of Soil Microbial Communities in Organic and Conventional Potato Farms. Plant Dis. 2010;94:1329–35.

43. Chaudhry V, Rehman A, Mishra A, Chauhan PS, Nautiyal CS. Changes in Bacterial Community Structure of Agricultural Land Due to Long-Term Organic and Chemical Amendments. Microb. Ecol. 2012;64:450–60.

44. Schneider S, Hartmann M, Enkerli J, Widmer F. Fungal community structure in soils of conventional and organic farming systems. Fungal Ecol. 2010;3:215–24.

45. Upchurch R, Chiu CY, Everett K, Dyszynski G, Coleman DC, Whitman WB. Differences in the composition and diversity of bacterial communities from agricultural and forest soils. Soil Biol. Biochem. 2008;40:1294–305.

46. Drijber RA, Doran JW, Parkhurst AM, Lyon DJ. Changes in soil microbial community structure with tillage under long-term wheat- fallow management. Soil Biol. Biochem. 2000;32:1419–1430.

47. Wang Z, Chen Q, Liu L, Wen X, Liao Y. Responses of soil fungi to 5-year conservation tillage treatments in the drylands of northern China. Appl. Soil Ecol. 2016;101:132–40.

48. Francioli D, Schulz E, Lentendu G, Wubet T, Buscot F, Reitz T. Mineral vs. organic amendments: Microbial community structure, activity and abundance of agriculturally relevant microbes are driven by long-term fertilization strategies. Front. Microbiol. 2016;7:10.3389/fmicb.2016.01446.

49. Zhen Z, Liu H, Wang N, Guo L, Meng J, Ding N, et al. Effects of manure compost application on soil microbial community diversity and soil microenvironments in a temperate cropland in China. PLoS One. 2014;9:e108555.

50. Lentendu G, Wubet T, Chatzinotas A, Wilhelm C, Buscot F, Schlegel M. Effects of long-term differential fertilization on eukaryotic microbial communities in an arable soil: A multiple barcoding approach. Mol. Ecol. 2014;23:3341–55.

51. Bulgarelli D, Garrido-Oter R, Münch PC, Weiman A, Dröge J, Pan Y, et al. Structure and function of the bacterial root microbiota in wild and domesticated barley. Cell Host Microbe. 2015;17:392–403.

52. Chaparro JM, Badri D V, Vivanco JM. Rhizosphere microbiome assemblage is affected by plant development. ISME J. 2014;8:790–803.

53. Haney CH, Samuel BS, Bush J, Ausubel FM. Associations with rhizosphere bacteria can confer an adaptive advantage to plants. Nat. Plants. 2015;1:1–9.

54. Bonito G, Reynolds H, Robeson MS, Nelson J, Hodkinson BP, Tuskan G, et al. Plant host and soil origin influence fungal and bacterial assemblages in the roots of woody plants. Mol. Ecol. 2014;23:3356–70.

55. Jumpponen A, Johnson LC. Can rDNA analyses of diverse fungal communities in soil and roots detect effects of environmental manipulations--a case study from tallgrass prairie. Mycologia. 2005;97:1177–94.

56. Ernest SKM, Brown JH. Homeostasis and compensation: The role of species and resources in ecosystem stability. Ecology. 2001;82:2118–32.

57. Hartmann M, Widmer F. Community structure analyses are more sensitive to differences in soil bacterial communities than anonymous diversity indices. Appl. Environ. Microbiol. 2006;72:7804–12.

58. Wu T, Chellemi DO, Martin KJ, Graham JH, Rosskopf EN. Discriminating the effects of agricultural land management practices on soil fungal communities. Soil Biol. Biochem. 2007;39:1139–55.

59. Ziemer CJ. Newly cultured bacteria with broad diversity isolated from eight-week continuous culture enrichments of cow feces on complex polysaccharides. Appl. Environ. Microbiol. 2014;80:574–85.

60. Shanks OC, Kelty CA, Archibeque S, Jenkins M, Newton RJ, McLellan SL, et al. Community structures of fecal bacteria in cattle from different animal feeding operations. Appl. Environ. Microbiol. 2011;77:2992–3001.

61. Verbarg S, Göker M, Scheuner C, Schumann P, Stackebrandt E. The Families Erysipelotrichaceae emend., Coprobacillaceae fam. Nov., and Turicibacteraceae fam. Nov. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes: Firmicutes and Tenericutes. 4th ed. Berlin-Heidelberg: Springer-Verlag; 2014. p. 79–105.

62. Goswami RS, Kistler HC. Heading for disaster: Fusarium graminearum on cereal crops. Mol. Plant Pathol. 2004;5:515–25.

63. Mercado Vergnes D, Renard ME, Duveiller E, Maraite H. Identification of Alternaria spp. on wheat by pathogenicity assays and sequencing. Plant Pathol. 2006;55:585–93.

64. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 2013;31:814–21.

65. Schlaeppi K, Bulgarelli D. The Plant Microbiome at Work. Mol. Plant Microbe Interact. 2015;28:212–7.

66. Shade A, Peter H, Allison SD, Baho DL, Berga M, Bürgmann H, et al. Fundamentals of microbial community resistance and resilience. Front. Microbiol. 2012;3:1–19.

67. Berry D, Widder S. Deciphering microbial interactions and detecting keystone species with co-occurrence networks. Front. Microbiol. 2014;5:1–14.

68. Ma B, Wang H, Dsouza M, Lou J, He Y, Dai Z, et al. Geographic patterns of co-occurrence network topological features for soil microbiota at continental scale in eastern China. ISME J. 2016;10:1891–901.

69. Weiss S, Van Treuren W, Lozupone C, Faust K, Friedman J, Deng Y, et al. Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. ISME J. 2016. https://doi.org/10.1669-81.

70. Wolfert S, Ge L, Verdouw C, Bogaardt MJ. Big Data in Smart Farming - A review. Agric. Syst. 2017;153:69–80.

71. Busby PE, Soman C, Wagner MR, Friesen ML, Kremer J, Bennett A, et al. Research priorities for harnessing plant microbiomes in sustainable agriculture. PLOS Biol. 2017;15:e2001793.

72. Chelius MK, Triplett EW. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. 2001;41:252–63.

73. Bodenhausen N, Horton MW, Bergelson J. Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. PLoS One. 2013;8:e56329.

74. Ihrmark K, Bödeker ITM, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, et al. New primers to amplify the fungal ITS2 region--evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiol. Ecol. 2012;82:666–77.

75. White T, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: Innis M, Gelfland D, Sninsky J, White T, editors. PCR Protoc. A Guid. to Methods Appl. San Diego, CA: Academic Press; 1990. p. 315–22.

76. Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets. Bioinformatics. 2011;27:863–4.

77. Magoč T, Salzberg SL. FLASH: Fast Length Adjustment of Short Reads to Improve Genome Assemblies. Bioinformatics. 2011;21:2957–63.

78. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal. 2011;17:10–2.

79. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods. 2013;10:996–8.

80. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27:2194–200.

81. Reddy TBK, Thomas AD, Stamatis D, Bertsch J, Isbandi M, Jansson J, et al. The Genomes OnLine Database (GOLD) v.5: a metadata management system based on a four level (meta)genome project classification. Nucleic Acids Res. 2015;43:D1099–106.

82. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–6.

83. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 2010;7:335–6.

84. Abarenkov K, Henrik Nilsson R, Larsson K-H, Alexander I, Eberhardt U, Erland S, et al. The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytol. 2010;186:281–5.

85. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2015.

86. Faria JC, Jelihovschi EG, Allaman Bezerra I. TukeyC: Conventional Tukey Test. 2016.

87. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One. 2013;8:e61217.

88. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. vegan: Community Ecology Package. 2015.

89. Anderson MJ, Willis TJ. Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. Ecology. 2003;84:511–25.

90. Hervé M. RVAideMemoire: Diverse Basic Statistical and Graphical Functions. 2016.

91. De Cáceres M, Legendre P, Moretti M. Improving indicator species analysis by combining groups of sites. Oikos. 2010;119:1674–84.

92. Csárdi G, Nepusz T. The igraph software package for complex network research. InterJournal, Complex Syst. 2006;1695. http://igraph.org.

93. Clauset A, Newman MEJ, Moore C. Finding community structure in very large networks. Phys. Rev. E. 2004;70:66111.

Additional file 1

Cropping Practices Manipulate Abundance Patterns of Root and Soil Microbiome Members Paving the Way to Smart Farming

Supplementary Methods

Chemical soil analysis

We collected soil samples for chemical analysis in March 2014, before the application of fertilizers or weed control measures. We took 20 soil cores with a soil auger (ø 2.5 cm) in the inner 2 x 10 m of each subplot to a soil depth of 0-20 cm and combined them to one meta-sample per plot. The samples were then sieved at 2 mm and kept at 4ºC until analysis. Soil samples were analyzed for pH, organic and total C, total N, and soil texture, extracted with 1:10 ammonia-acetate-EDTA and determined according to the reference methods of the Swiss Federal Research Stations [1].

16S PCR and library preparation

The 16S amplicon library was generated using the PCR primers 799F [2] and 1193R [3]. The primers were extended at the 5`end with an error-tolerant barcode for multiplexed library sequencing (Supplementary Data S1). PCR reactions were performed on a iCycler instrument (BioRad, Hercules, CA, USA) using the 5PRIME Hot Master Mix PCR system (5 PRIME, Gaithersburg, MD USA) with the cycling conditions in Table S1. Each 20 µL reaction contained: 8 µL 5PRIME Hot Master Mix, 0.3 % BSA, 200 nM each primer, and 2 ng and 10 ng of DNA template for soil and root reactions respectively, and the remaining volume sterile distilled water. PCR reactions were conducted in quadruplicates and pooled together before inspecting 3 µL of each sample on a 1 % agarose gel at 90 V for 45 min for correct size and absence of contamination in non-template reactions. PCR reactions were then purified using the NucleoSpin Gel and PCR Clean up Kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The purified reactions were quantified using the same Picogreen assay described above and pooled in equal amounts (100 ng / sample), after which the library volume was reduced using a CentriVap centrifugal vacuum concentrator (Labconco Corp., Kansas City, MO, USA). The concentrated library was mixed with loading dye, split equally between 2 lanes of a 1.2 % agarose gel to separate the 'bacteria band' from the ~800 bp mitochondria product also produced by the primers. Bacteria bands were cut and these gel fragments purified using the kit described above, eluted in 50 µL of the

supplied elution buffer and measured using a Qubit assay (Agilent Technologies, Santa Clara, USA).

ITS PCR and library preparation

The ITS amplicon library was generated using the PCR primers fITS7 [4] and ITS4 [5]. The primers were extended at the 5`end with an error-tolerant barcode for multiplexed library sequencing (Supplementary Data S1). PCR reactions were performed on an iCycler instrument (BioRad, Hercules, CA, USA) using the DreamTaq PCR system (Thermo-Fisher Scientific, Waltham, MA, USA) with the cycling conditions in Table S1. Each 20 µL reaction contained: 10 µL DreamTaq PCR MasterMix (DreamTaq DNA Polymerase, 1x DreamTaq Buffer, 2 mM $MgCl_2^+$, 200 µM each dNTP), supplemental $MgCl_2^+$ to 2.75 mM, 0.3 % BSA, 500 nM of the forward primer, 200 nM of the reverse primer, 10 ng of DNA template for both soil and root samples, and the remaining volume sterile distilled water. PCR reactions were conducted in quadruplicates and pooled together before validation by gel electrophoresis. The reactions were quantified using a Picogreen assay and pooled in equal amounts (200 ng / sample). The volume of the pooled library was reduced using a CentriVap centrifugal vacuum concentrator (Labconco Corp., Kansas City, MO, USA), mixed with loading dye and subjected to separation on a 1.5% agarose gel. The bands between 300-500 bp were cut from the gel and purified with the NucleoSpin Gel and PCR Clean up Kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions, eluted in 50 µL of the supplied elution buffer, and the DNA quantified using a Qubit assay (Agilent Technologies, Santa Clara, USA).

Library sequencing

Preparation of the 16S and ITS amplicon libraries was conducted as follows: The TruSeq DNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) was used following the manufacturer's instructions. Briefly, the amplicon samples were end-repaired and polyadenylated. TruSeq adapters containing the index for multiplexing were ligated to the amplicon samples. The ligated samples were run on a 2% agarose gel and the desired fragment length was excised (50 bp +/- the target fragment length). DNA from the gel was purified with MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). Fragments containing TruSeq adapters on both ends were selectively enriched with PCR using 4 cycles. The quality and quantity of the enriched libraries were validated using Qubit and Tapestation (Agilent Technologies, Santa Clara, CA USA). The libraries were normalized to 4 nM in Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20. The library was sequenced on the Illumina MiSeq Personal Sequencer (Illumina, San Diego, USA) using a 600 cycle v3 Sequencing kit (Cat n° MS-102-

3003), paired-end 2x 300 cycle sequencing mode at the Functional Genomics Center Zurich (www.fgcz.ch).

Supplementary Results

Taxonomic profiles of soil and root bacterial and fungal communities

Differences between the soil and root microbiota were evident in the taxonomic profiles of both sample types. We noted 30 bacteria phyla present in soil samples, with Proteobacteria (39.3%), Actinobacteria (31.2%), and Firmicutes (6.3%) having the highest relative abundances (Fig. S3). We found 25 different phyla in root samples with Actinobacteria (41%), Proteobacteria (39.7%), and Bacteroidetes (10.7%) being most abundant. In fungal communities, soils contained at least six phyla, with abundant Ascomycota (72.2%), Basidiomycota (9.4%) and Zygomycota (4.9%). OTUs from unassigned phyla made up ~11.9% of fungi OTUs. In root samples, we also found OTUs from at least six phyla with abundant Ascomycota (80.6%) and Basidiomycota (16.2%). OTUs from unassigned phyla comprised ~3% of the community. We found that the proportion of sequences from the phylum Glomeromycota, which contain the arbuscular mycorrhizal fungi (AMF), was generally very low in both sample types (mean relative abundance of 1% in soil samples and 0.02% in root samples), confirming that the primer combination fITS7 - ITS4 is suboptimal to characterize AMF communities (Fig. S3). It is known that general fungal ITS primers poorly resolve and discriminate AMF taxa [6,7].

Taxonomic patterns of csOTUs

Cropping sensitive OTUs (*cs*OTUs) were identified based on indicator species analysis and using likelihood ratio tests. The 53 *cs*OTUs in the soil bacterial community (Fig. S6) comprised at least 11 phyla, with the majority of community sequences belonging to the Actinobacteria (25.4%), Proteobacteria (22.5%) and Firmicutes (18.4%). We noted that specific phyla tended to respond to specific management systems and tillage regimes. OTUs belonging to the Firmicutes favored organically managed plots (Fig. S7). Bacteroidetes OTUs tended towards higher mean abundances in no-till and reduced tillage treatments; whereas mean abundances of OTUs from the Acidobacteria and Verrucomicrobia were higher in the full-tillage treatments. OTUs from the Chloroflexi tended to favor the O-RT system. We also examined the taxonomic assignment and mean relative abundances of the individual *cs*OTUs across the four cropping systems (Fig. S8). We noted higher relative abundances of Firmicutes OTUs *bOTU36* (family *Erysipelotrichaceae*), *bOTU23, bOTU119* (both

Peptostreptococcaceae) and *bOTU341* (*Clostridiaceae*) in organically managed plots, consistent with patterns seen at the phylum level (Figs. S7-S9). We observed similar patterns for OTUs from the phylum Acidobacteria (*bOTU806*, *bOTU885*, *bOTU238*, *bOTU651*, family unassigned), which were consistently more abundant in plots receiving intensive tillage (Figs. S8, S9).

The bulk soil fungal community comprised 70 *cs*OTUs (Fig. S6) classified into at least six different phyla, with Ascomycota (81.2% of sequences) unassigned (8.5%) and Basidiomycota (5.5%) being the most abundant (Fig. S7). We observed a number of known Ascomycota OTUs, possibly belonging to pathogenic fungi, that were abundant in C-NT system (*fOTU57*, family *Nectriaceae*) and organically managed plots (*fOTU32 Nectriaceae; fOTU25 and fOTU1628 Sporormiaceae*) (Figs. S8, S10). We also noted that a single OTU from the phylum Glomeromycota (*fOTU980*, family *Diversisporaceae*) was absent in C-IT samples and enriched in O-IT.

In root bacterial communities, the 63 *cs*OTUs (Fig. S6) were classified into ten different phyla, with Actinobacteria, Proteobacteria and Firmicutes having the highest relative abundances (73.4%, 13,2%, and 9.5% of sequences, respectively; Fig. S7). Across the four cropping systems, OTUs from the Actinobacteria were equally well represented. OTUs from the Proteobacteria and Bacteroidetes, were more abundant in reduced and no-tillage plots. Like in the soil bacterial community, the Firmicutes were generally more abundant in root samples from organically managed plots. This appeared to be driven by the increased abundance of several OTUs from the family *Peptostreptococcaceae* (*bOTU23, bOTU119), Clostridiaceae* (*bOTU341*), *Erysipelotrichaceae* (*bOTU36*), and *Lachnospiraceae* (*bOTU1403*), a family that was exclusive to organically managed plots. (Figs. S8, S11).

The 36 *cs*OTUs (Fig. S6) in root fungal communities were classified into at least three phyla. Most sequences belonged to the Ascomycota (75.9%), followed by unassigned phyla (14.1%), and Basidiomycota (9.8%; Fig. S7). We noted that OTUs from the Ascomycota favored the C-NT system and, to a lesser extent, the organically managed plots. The O-RT system supported a higher abundance of OTUs belonging to unassigned phyla and the Basidomycota. Many of the farming system sensitive OTUs were unassigned at lower taxonomic levels (Fig. S8). However, in the Ascomycota, *fOTU63* (*Pleosporaceae)* and *fOTU97* (*Phaeosphaeriaceae)* were abundant in the C-NT system, while the *Psathyrellaceae fOTU86* was abundant in the O-RT system (Figs. S8, S12). We also noted a number of OTUs from the family *Lasiosphaeriaceae* with higher mean abundances in the O-IT treatment.

Supplementary Discussion

Cropping system effects on soil microbial communities

We found significant effects of cropping system on soil microbial communities, explaining approximately 30% of the total variation in both bacteria and fungi (Fig. 2). More specifically, bacterial communities were more strongly separated by the different tillage regimes rather than by management type, with the biggest differences between intensive tillage samples and those receiving less intensive tillage (Table S4). This finding is somewhat unexpected given that earlier work has shown that the addition of manure, as is the case in the organically managed plots, can result in substantial shifts in soil bacterial community [8–11]. Moreover, bacteria are generally thought to be relatively unaffected by tillage practices, given their small cell size and constrained dispersal and are therefore, less likely to be affected by the homogenization of soil microsites [12,13].

It has also been suggested that bacteria introduced into soils from manure amendments do not become prominent [9] and that any bacterial community compositional shifts as a result of manure additions tend to diminish over time [8–10]. However, these results would seemingly conflict with a number of recent studies that have profiled microbial communities in soils receiving inorganic and organic fertilizer and found substantial differences between the two fertilizer regimes [14–17]. For example, Hartmann *et al.,* [15] profiled soil microbial communities from a long-term (>20 years) Swiss agricultural experiment comparing five different management systems receiving either mineral fertilizer or farm yard manure. They found that the application of farm yard manure was the primary driving force behind bacterial community dissimilarity. Thus, we hypothesize our findings could be attributed to two reasons. First, because we collected soil samples over two months after the final application of manure in the organically managed plots, any initial changes to the bacterial community may have largely disappeared by the time the samples were collected. Second, the abovementioned studies reporting manure induced shifts in bacterial community composition were all conducted on long-term agricultural trials under decades of manure amendment. Although the entire FAST experimental site has been under organic management since 2002, the cropping treatments were only established for FAST II in 2010 [18]. Therefore, our results may be indicative of the relatively short period of manure amendments at the site.

In contrast to soil bacteria, constrained ordinations of soil fungal communities revealed that differences between conventional and organic management types explained most of the variation (Fig. 2). Despite the relatively short term management of the FAST site, our results

are more in accordance with previous studies on long-term (>20 years) agricultural trials that reported significant effects of organic management with manure fertilization on soil fungal community composition [15,19]. Studies on soil communities subjected to organic management with manure additions over the short term (typically less than 10 years) have tended to report no significant differences in fungal community structure between manure amended and non-amended soils [20,21]. However, these shorter-term studies relied on older molecular tools, which may be less precise in capturing subtle community shifts compared to amplicon sequencing [19].

Nevertheless, there is evidence that the addition of manure to soils represents an input of external microbes that could affect strong changes in the diversity and composition of both bacterial and fungal communities over the course of a growing season [19,22]. With this in mind, our results highlight the need for future studies to assess the temporal variability in soil communities receiving external microbial inputs, such as manure. Sampling at multiple time points, including before manure application, would shed light on the dynamics of the bacterial and fungal communities during the course of the growing season. This could help to improve estimates of microbial α-diversity, which have been shown to exhibit greater temporal variability than across different land use types [23]. Furthermore, future studies would benefit from the inclusion of manure samples in high-throughput sequencing runs for the direct identification of manure-derived bacteria and fungi OTUs based on sequence similarity.

We found that an increase in tillage intensity from reduced tillage to intensive tillage resulted in significantly different soil fungal communities in organically managed plots; whereas the same was not observed between no-till and intensive tillage samples in conventional plots (Table S4). This suggests that tillage effects on soil fungal communities may depend on other factors, such as management type. Other previous work on the effects of soil disturbance events on soil fungi have often focused on AMF as a group of fungi sensitive to increasing tillage intensity [24–26]. However, we are unable to draw conclusions about effects of tillage on AMF communities at the FAST site due to very low abundances of AMF sequences (Fig. S3). It is generally thought that tillage affects soil AMF communities through physical destruction of dense hyphal networks [27]. Such mechanisms of physical disturbance are also thought to influence communities of general soil fungi, and therefore less soil disturbance and more heterogeneous resource distribution, common of no till and reduced tillage systems, may promote fungal communities [28]. Many hypotheses about the effects of tillage on fungal communities also focus on indirect effects, namely that tillage influences edaphic factors like soil organic carbon content [29,30] and soil nutrient pools like extractable

P [31], which have been shown to influence soil fungal community composition. Similarly, our unconstrained ordination analyses revealed that differences in pH explained approximately 24% and 27% of community variation in the soil bacterial and fungal communities, respectively (Fig. S4). These results are generally consistent with previous findings showing soil pH as a significant driver of primarily bacterial community composition [32,33], but also of fungi [34]. However, it is important to stress that our findings were less the result of a true pH gradient across multiple samples and more the result of a low pH value in one subplot.

Cropping system effects on microbial α-diversity

We have assessed the effects of cropping systems on observed bacteria and fungi OTU richness in both soil and root samples, confirming that soils were more diverse than root microbial communities [35,36]. With respect to the effects of cropping system, we found the soil bacteria and fungi tended to be richest in the O-IT system (Fig. S5, Table S3). These observations are in accordance with previous studies reporting higher soil microbial richness in organically managed compared to conventionally managed soils (bacteria: 29, 50, 51; fungi: 29, 52). However, there are also studies reporting no differences between conventional and organic managements [40,41]. We speculate that timing differences between application and sampling might explain conflicting results, in that any enhanced diversity effects might disappear in the time span between manure application and sampling.

The effects of differential soil managements on the root microbes appear to vary depending on the root compartment analyzed. Edwards *et al.,* [42] found differences in bacteria α-diversity in the rhizosphere but not rhizoplane and endosphere compartments when comparing samples from conventional and organically managed cropping systems. Also Seghers *et al.,* [43] found no difference in maize root endophyte richness (bacteria and fungi) in samples taken from conventionally and organically managed plots. Soil management seems to affect microbial communities to a lesser extent the more intimately they associate with their host plant. We think that our root sampling method without physical (no sonication) or chemical (no detergent or bleach) separation from the rhizosphere compartment yields a rather low-intimacy type of compartment, and we expected to find impacts by soil management. Indeed, we found effects of cropping practices on observed root OTU richness. We found significantly higher richness in in O-IT plots compared to conventionally managed plots for the bacteria (Fig. S5; Table S3).

Taken together, we find enhanced richness in root and soil microbiota in O-IT systems. We think that the application of animal manure as fertilizer coupled with structural disturbance presents a likely explanation for the enhanced diversity in organic intensive tillage systems.

Supplementary References

1. Eidgenössische Forschungsanstalten FAL, RAC F. Schweizerische Referenzmethoden der Eidgenössischen landwirtschaftlichen Forschungsanstalten. Zürich: Eidg. Forschungsanstalt für Landwirtschaftlichen Pflanzenbau, FAP; 1996.

2. Chelius MK, Triplett EW. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. 2001;41:252–63.

3. Bodenhausen N, Horton MW, Bergelson J. Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. PLoS One. 2013;8:e56329.

4. Ihrmark K, Bödeker ITM, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, et al. New primers to amplify the fungal ITS2 region--evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiol. Ecol. 2012;82:666–77.

5. White T, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: Innis M, Gelfland D, Sninsky J, White T, editors. PCR Protoc. A Guid. to Methods Appl. San Diego, CA: Academic Press; 1990. p. 315–22.

6. Stockinger H, Krüger M, Schüßler A. DNA barcoding of arbuscular mycorrhizal fungi. New Phytol. 2010;187:164.

7. Kohout P, Sykorova Z, Ctvrtlikkova M, Rydlova J, Suda J, Vohnik M, et al. Surprising spectra of root-associated fungi in submerged aquatic plants. FEMS Microbiol. Ecol. 2012;80:216–35.

8. Hammesfahr U, Heuer H, Manzke B, Smalla K, Thiele-Bruhn S. Impact of the antibiotic sulfadiazine and pig manure on the microbial community structure in agricultural soils. Soil Biol. Biochem. 2008;40:1583–91.

9. Heuer H, Focks A, Lamshöft M, Smalla K, Matthies M, Spiteller M. Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. Soil Biol. Biochem. 2008;40:1892–900.

10. Jechalke S, Focks A, Rosendahl I, Groeneweg J, Siemens J, Heuer H, et al. Structural and functional response of the soil bacterial community to application of manure from difloxacin-treated pigs. FEMS Microbiol. Ecol. 2014;87:78–88.

11.Jangid K, Williams MA, Franzluebbers AJ, Sanderlin JS, Reeves JH, Jenkins MB, et al. Relative impacts of land-use, management intensity and fertilization upon soil microbial community structure in agricultural systems. Soil Biol. Biochem. 2008;40:2843–53.

12. Strickland MS, Rousk J. Considering fungal:bacterial dominance in soils – Methods, controls, and ecosystem implications. Soil Biol. Biochem. 2010;42:1385–95.

13. Hendrix P, Parmelee R, Crossley Jr D, Coleman D, Odum E, Groffman P. Detritus Food Webs in Conventional and No- Tillage Agroecosystems. Bioscience. 1986;36:374–80.

14. Li F, Chen L, Zhang J, Yin J, Huang S. Bacterial Community Structure after Long-term Organic and Inorganic Fertilization Reveals Important Associations between Soil Nutrients and Specific Taxa Involved in Nutrient Transformations. Front. Microbiol. 2017;8:10.3389/fmicb.2017.00187.

15. Hartmann M, Frey B, Mayer J, Mäder P, Widmer F. Distinct soil microbial diversity under long-term organic and conventional farming. ISME J. 2014;9:1177–1194.

16. Ding J, Jiang X, Ma M, Zhou B, Guan D, Zhao B, et al. Effect of 35 years inorganic fertilizer and manure amendment on structure of bacterial and archaeal communities in black soil of northeast China. Appl. Soil Ecol. 2016;105:187–95.

17. Francioli D, Schulz E, Lentendu G, Wubet T, Buscot F, Reitz T. Mineral vs. organic amendments: Microbial community structure, activity and abundance of agriculturally relevant microbes are driven by long-term fertilization strategies. Front. Microbiol. 2016;7:10.3389/fmicb.2016.01446.

18. Wittwer RA, Dorn B, Jossi W, van der Heijden MGA. Cover crops support ecological intensification of arable cropping systems. Sci. Rep. 2017;7:10.1038/srep41911.

19. Lentendu G, Wubet T, Chatzinotas A, Wilhelm C, Buscot F, Schlegel M. Effects of long-term differential fertilization on eukaryotic microbial communities in an arable soil: A multiple barcoding approach. Mol. Ecol. 2014;23:3341–55.

20. Marschner P, Kandeler E, Marschner B. Structure and function of the soil microbial community in a long-term fertilizer experiment. Soil Biol. Biochem. 2003;35:453–61.

21. Lejon DPH, Sebastia J, Lamy I, Chaussod R, Ranjard L. Relationships between soil organic status and microbial community density and genetic structure in two agricultural soils submitted to various types of organic management. Microb. Ecol. 2007;53:650–63.

22. Zhen Z, Liu H, Wang N, Guo L, Meng J, Ding N, et al. Effects of manure compost application on soil microbial community diversity and soil microenvironments in a temperate cropland in China. PLoS One. 2014;9:e108555.

23. Lauber CL, Ramirez KS, Aanderud Z, Lennon J, Fierer N. Temporal variability in soil microbial communities across land-use types. ISME J. 2013;7:1641–50.

24. Jansa J, Mozafar A, Anken T, Ruh R, Sanders IR, Frossard E. Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza. 2002;12:225–34.

25. Drijber RA, Doran JW, Parkhurst AM, Lyon DJ. Changes in soil microbial community structure with tillage under long-term wheat- fallow management. Soil Biol. Biochem. 2000;32:1419–1430.

26. Alguacil M., Lumini E, Roldan A, Salinas-Garcia J, Bonafante P, Bianciotto V. The impact of tillage practices on arbuscular mycorrhizal fungal diversity in subtropical crops. Ecol. Appl. 2008;18:527–36.

27. Verbruggen E, Kiers TE. Evolutionary ecology of mycorrhizal functional diversity in agricultural systems. Evol. Appl. 2010;3:547–60.

28. Helgason BL, Walley FL, Germida JJ. Fungal and bacterial abundance in long-term no-till and intensive-till soils of the Northern Great Plains. Soil Sci. Soc. Am. J. 2009;73:120–7.

29. Wang Z, Chen Q, Liu L, Wen X, Liao Y. Responses of soil fungi to 5-year conservation tillage treatments in the drylands of northern China. Appl. Soil Ecol. 2016;101:132–40.

30. Liu J, Sui Y, Yu Z, Shi Y, Chu H, Jin J, et al. Soil carbon content drives the biogeographical distribution of fungal communities in the black soil zone of northeast China. Soil Biol. Biochem. 2015;83:29–39.

31. Lauber CL, Strickland MS, Bradford MA, Fierer N. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol. Biochem. 2008;40:2407–15.

32. Fierer N, Jackson RB. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. U. S. A. 2006;103:626–31.

33. Lauber CL, Hamady M, Knight R, Fierer N. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Appl. Environ. Microbiol. 2009;75:5111–20.

34. Rousk J, Bååth E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, et al. Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J. 2010;4:1340–51.

35. Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 2013;64:807–38.

36. Jumpponen A, Johnson LC. Can rDNA analyses of diverse fungal communities in soil and roots detect effects of environmental manipulations--a case study from tallgrass prairie. Mycologia. 2005;97:1177–94.

37. Chaudhry V, Rehman A, Mishra A, Chauhan PS, Nautiyal CS. Changes in Bacterial Community Structure of Agricultural Land Due to Long-Term Organic and Chemical Amendments. Microb. Ecol. 2012;64:450–60.

38. Lupatini M, Korthals GW, de Hollander M, Janssens TKS, Kuramae EE. Soil Microbiome Is More Heterogeneous in Organic Than in Conventional Farming System. Front. Microbiol. 2017;7:10.3389/fmicb.2016.02064.

39. Verbruggen E, Röling WFM, Gamper HA, Kowalchuk GA, Verhoef HA, van der Heijden MGA. Positive effects of organic farming on below-ground mutualists : large-scale comparison of mycorrhizal fungal communities in agricultural soils. New Phytol. 2010;186:968–79.

40. Liu B, Tu C, Hu S, Gumpertz M, Ristaino JB. Effect of organic, sustainable, and conventional management strategies in grower fields on soil physical, chemical, and biological factors and the incidence of Southern blight. Appl. Soil Ecol. 2007;37:202–14.

41. Hartmann M, Widmer F. Community structure analyses are more sensitive to differences in soil bacterial communities than anonymous diversity indices. Appl. Environ. Microbiol. 2006;72:7804–12.

42. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, et al. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc. Natl. Acad. Sci. 2015;112:E911–20.

43. Seghers D, Wittebolle L, Top EM, Verstraete W, Siciliano S. Impact of agricultural practices on the Zea mays L. endophytic community. Appl. Environ. Microbiol. 2004;70:1475–82.

Supplementary Figures

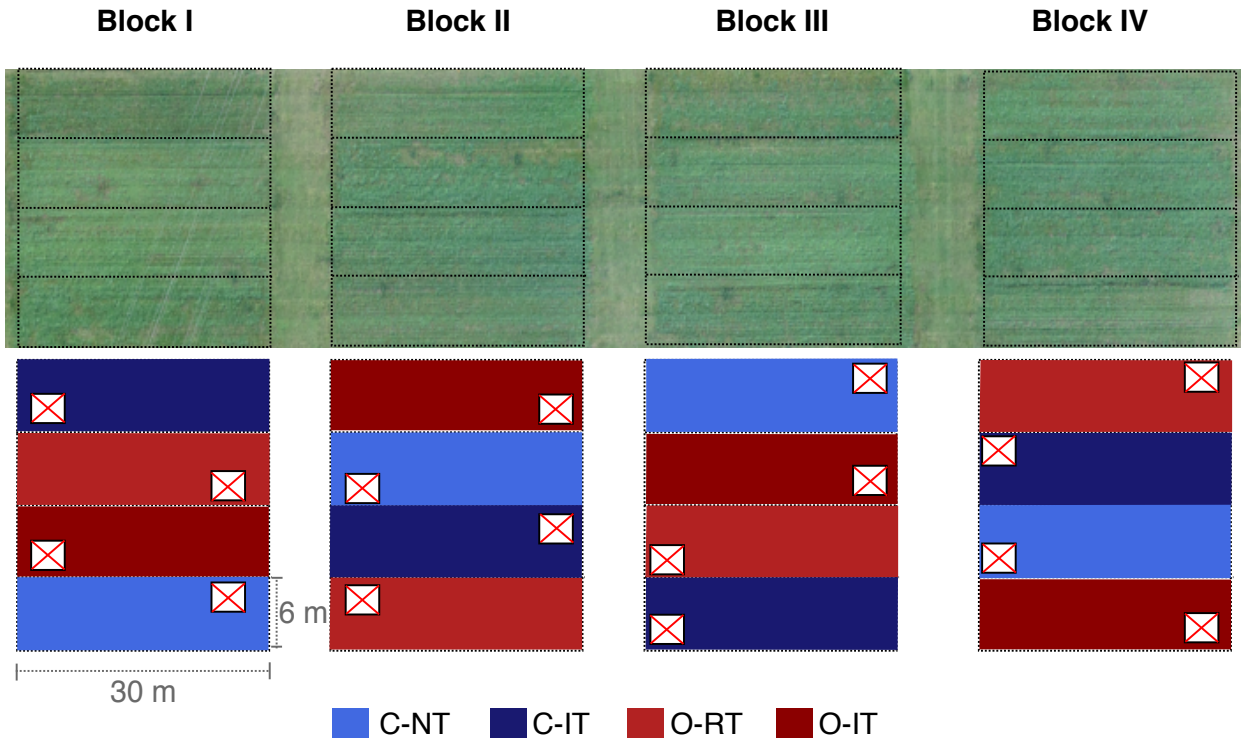

Figure S1: Experimental layout of the FAST experiment. The top panel is an aerial photograph of the four blocks at experimental site with individual plots outlined in dashed lines. The cropping system applied to each plot is indicated in the colored bottom panel. White boxes marked with X's indicate the approximate sampling location of root and soil samples within each plot.

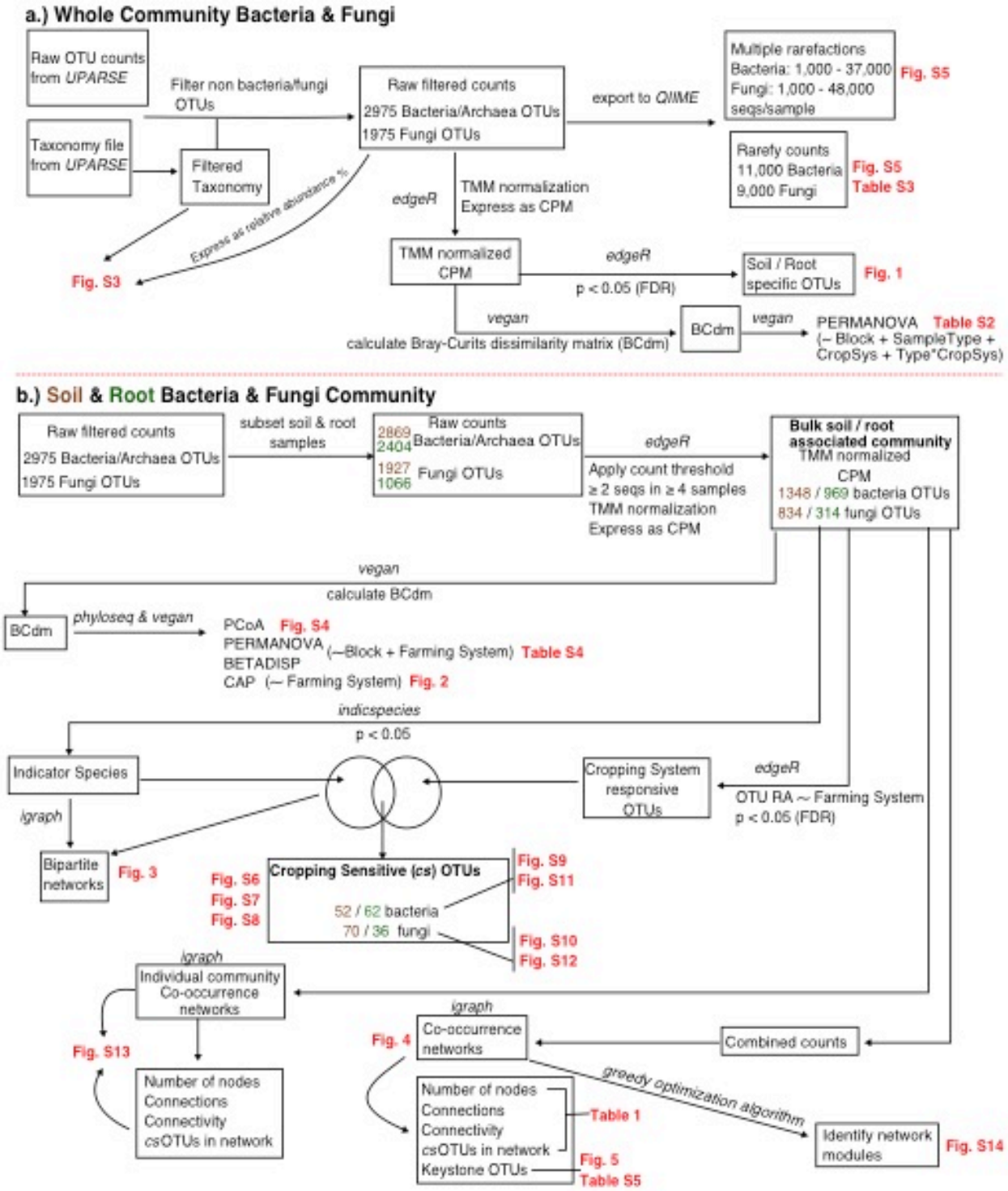

Figure S2: Schematic flow diagram of analysis steps for **(a)** whole community **(b)** soil and root bacterial and fungal communities. Numbers in brown refer to soil samples. Numbers in green refer to root samples. The figures generated as the output from each step are indicated in red.

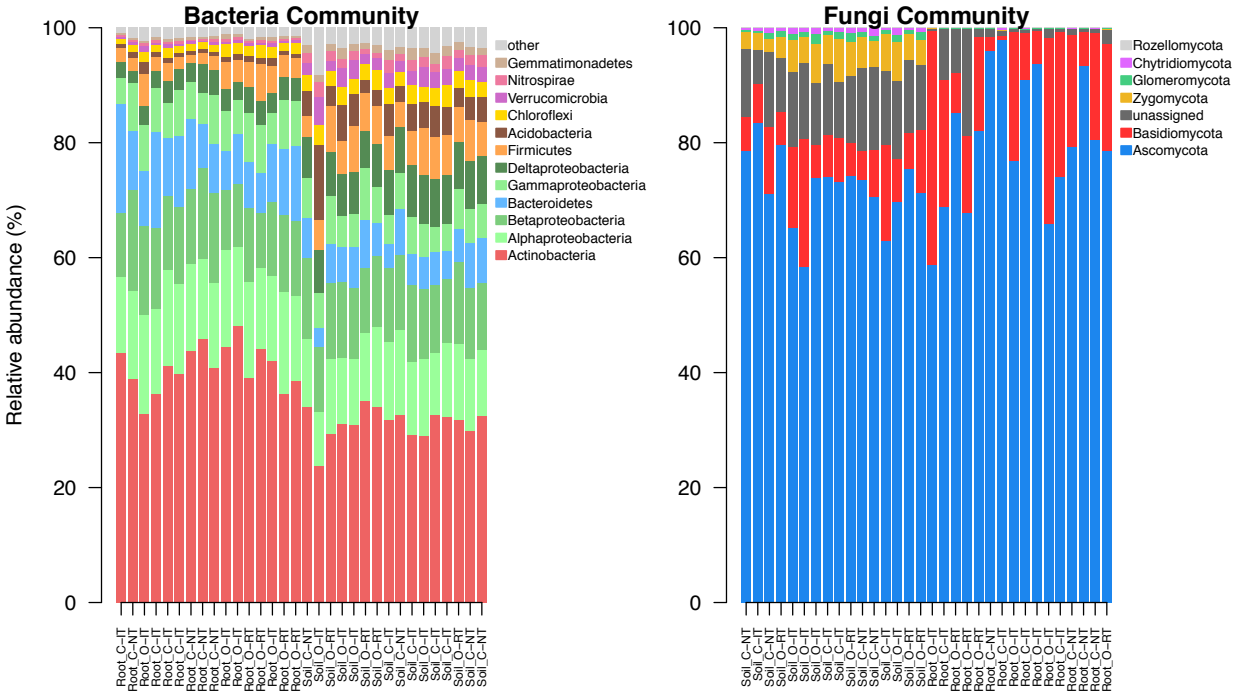

Figure S3: Taxonomic profiles of bacteria and fungi communities at phylum level. Bacteria phyla with relative abundances lower than 1% were summarized with 'other'. The x-axis sample order reflects a clustering by Bray-Curtis dissimilarities using the *hclust* function in R with method "average".

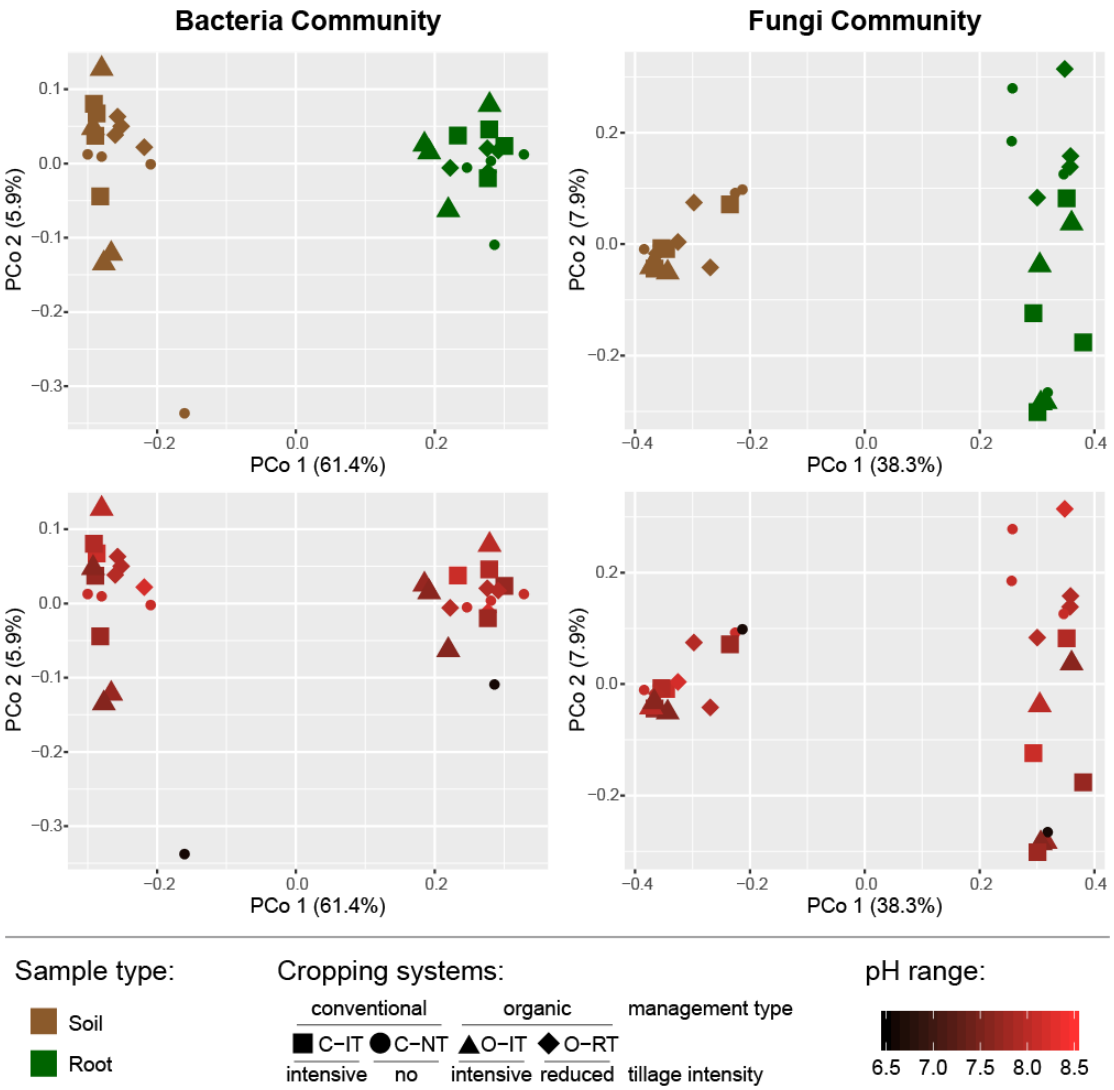

Figure S4: Unconstrained PCoA ordinations of bacteria (left) and fungi (right). Sample type presented the major driver of community variation. Percentage of variation given on each axis refers to the explained fraction of total variation in the community. Upper and lower panels are colored by sample type (root vs. soil samples) and soil pH values, respectively. Symbols refer to the different cropping systems.

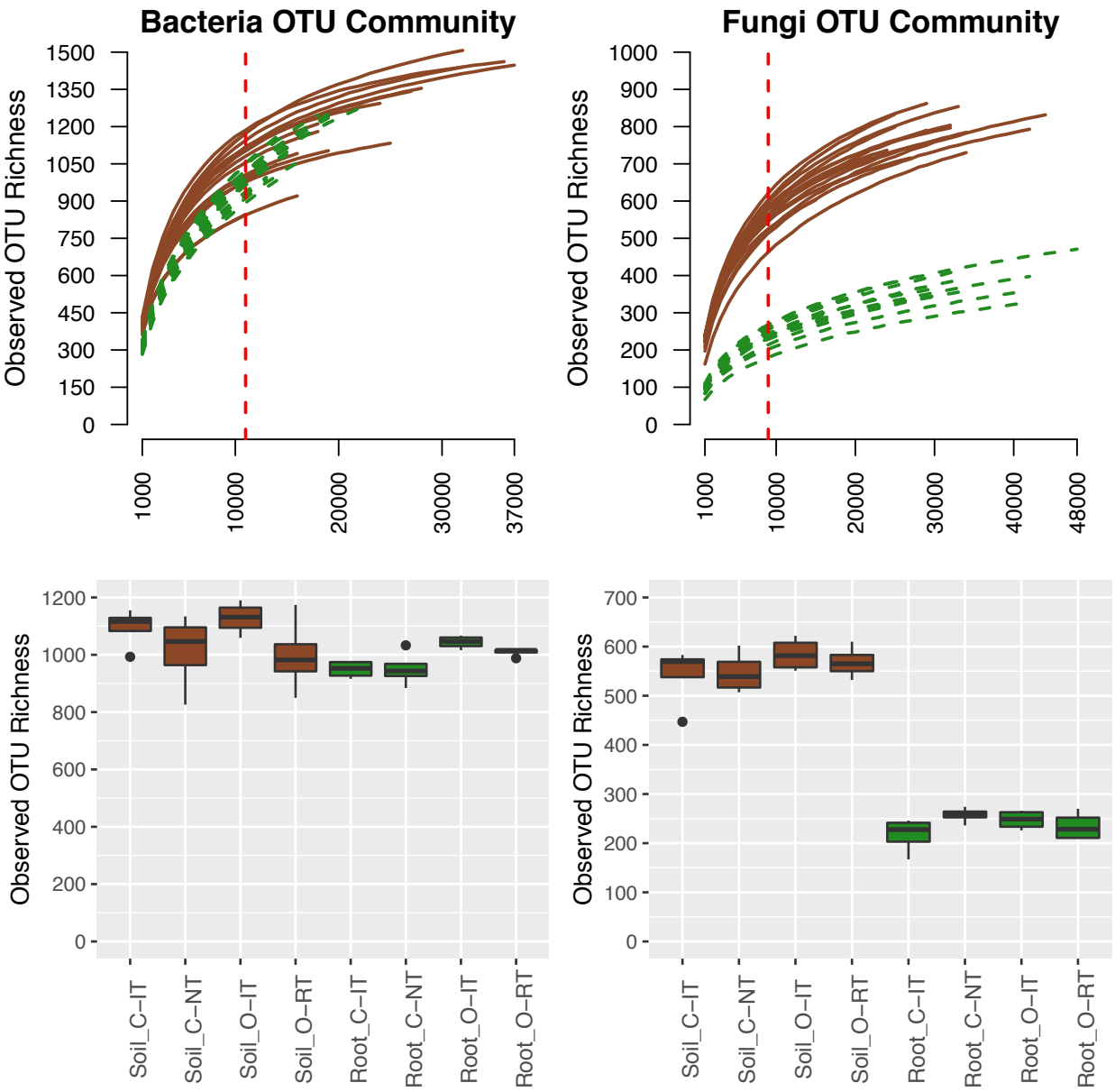

Figure S5: Rarefaction curves for bacteria and fungi observed OTU richness. Brown lines indicate soil samples, and green lines indicate root samples. The dashed red line indicates the selected rarefaction depth used to generate the box plots below each curve, 11,000 seqs/sample and 9,000 sequences per sample for bacterial and fungal communities, respectively. The boxplots show the effective OTU richness at the respective rarefaction depths for bacteria and fungi. *X* axis labels indicate the sample type and cropping system of each box, which are colored by sample type. Results of the t-tests, 2-way ANOVA and subsequent post-hoc tests, if applicable, are given in Table S3.

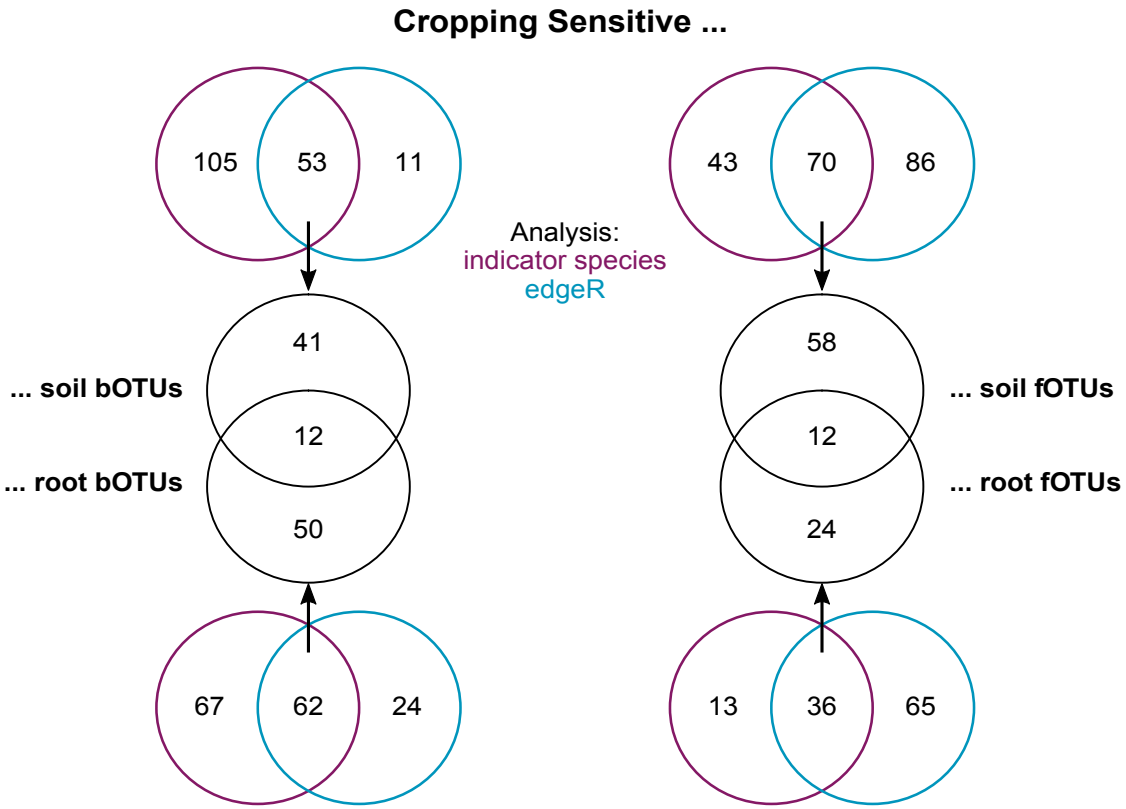

Figure S6: Defining cropping sensitive bacteria (b-) and fungi (f-) OTUs in soil and root samples. Venn diagrams show the number of OTUs responding to cropping practices identified with indicator species analysis (purple) and by edgeR (cyan). OTUs identified by both methods were defined as cropping sensitive OTUs (*cs*OTUs).

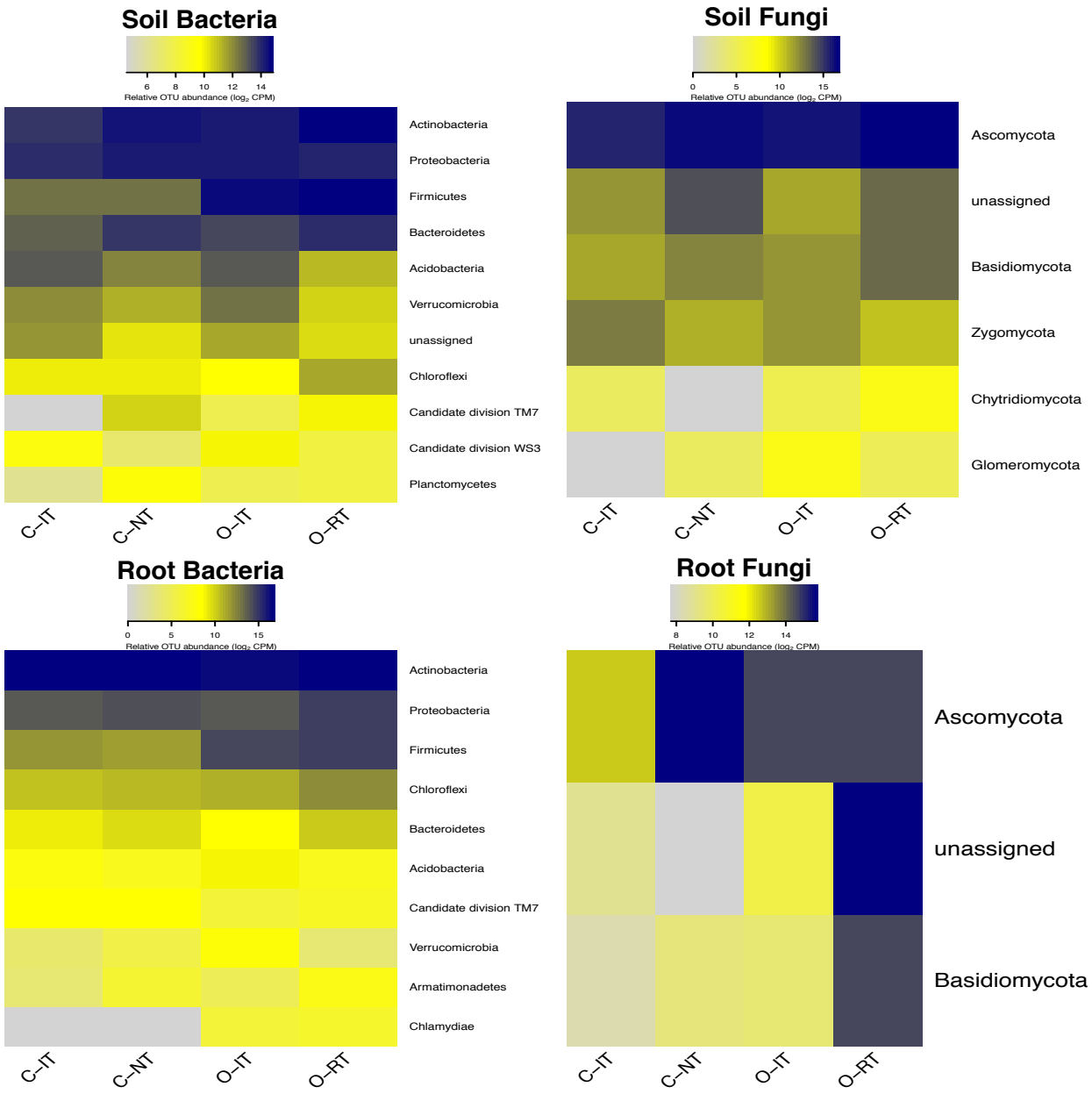

Figure S7: Mean relative abundances (counts per million, CPM; log2 scale) of cropping sensitive OTUs (as defined in Fig. S6, summarized at phylum level) across cropping systems for soil bacteria, soil fungi, root bacteria, and root fungi.

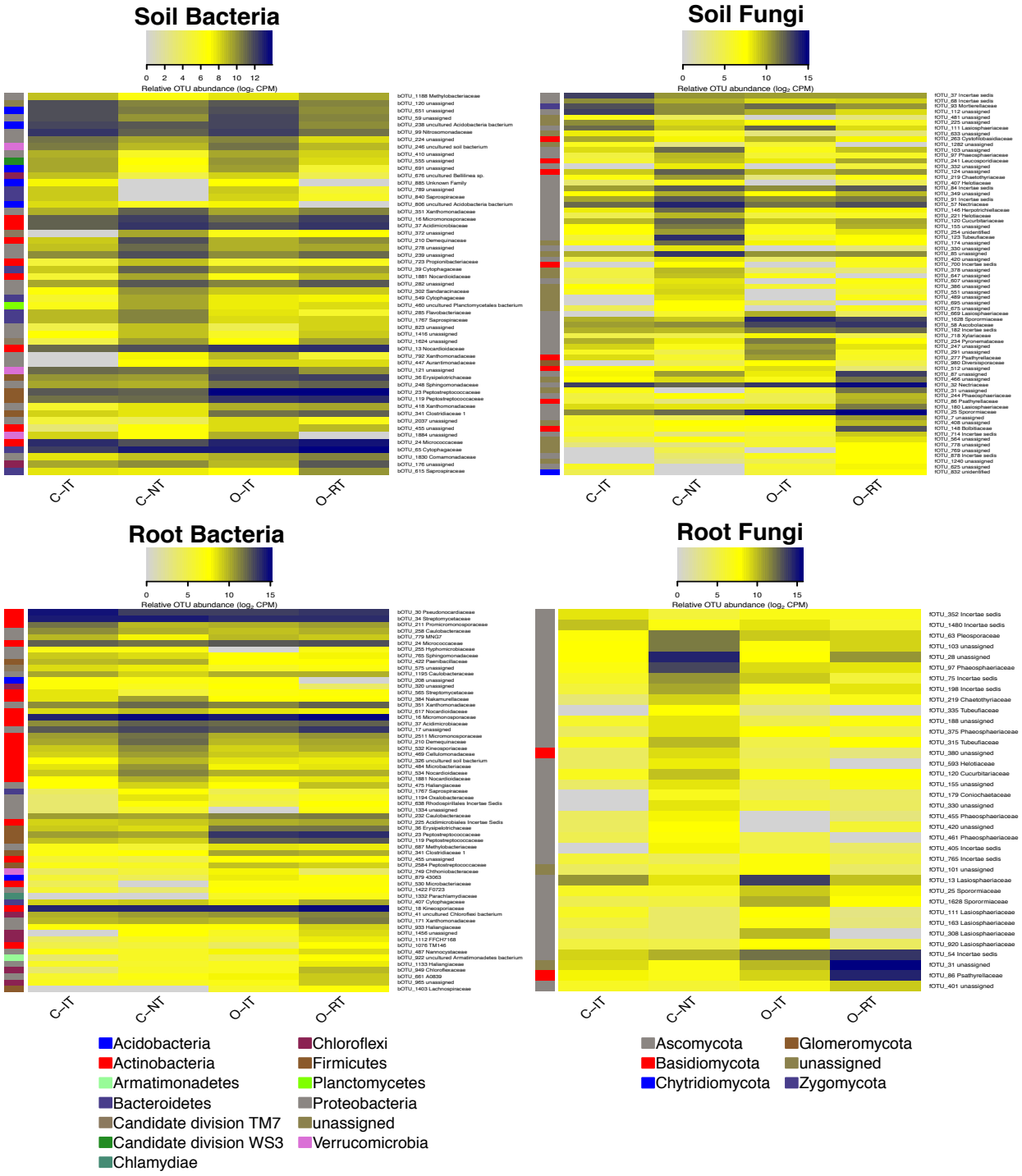

Figure S8: Mean relative abundances (counts per million, CPM; log2 scale) of cropping sensitive OTUs identified by indicator species analysis and *edgeR* (see Fig. S6). OTUs are labeled with their family level taxonomy assignment, with the phylum level taxonomy assignment indicated by the colored bars.

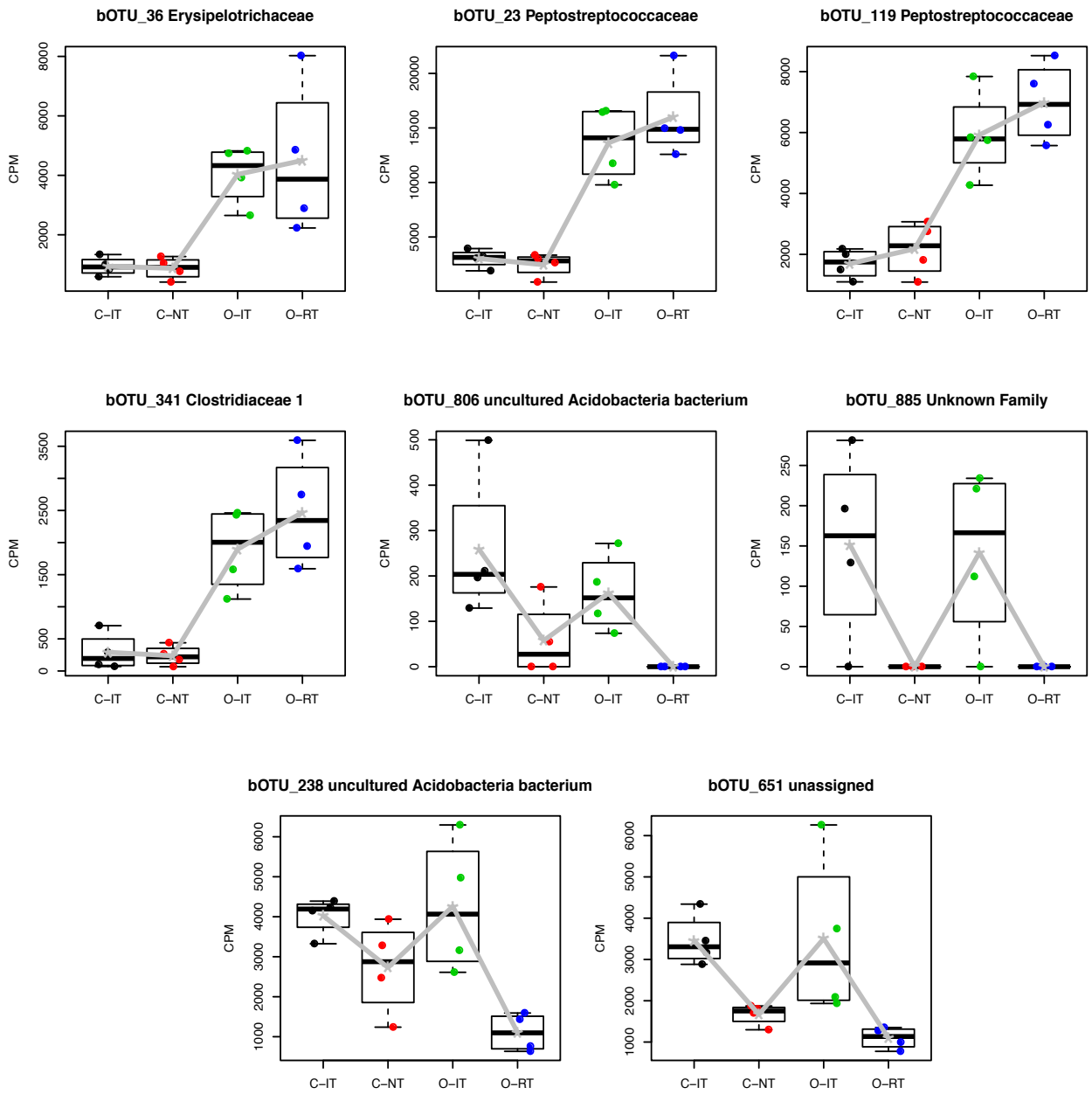

Figure S9: Relative abundances (counts per million, CPM) of abundant cropping sensitive bacteria (bOTUs) in soil. Means within each cropping system are indicated in gray stars.

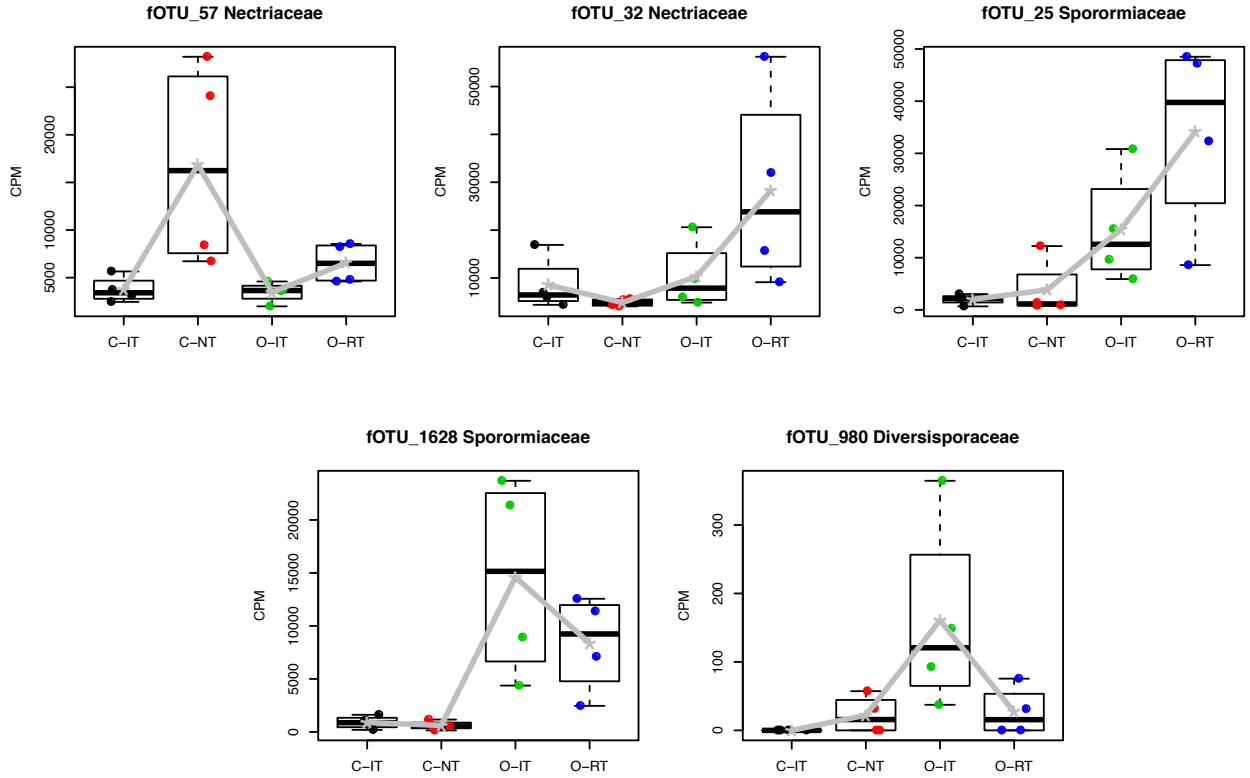

Figure S10: Relative abundances (counts per million, CPM) of abundant cropping sensitive fungi (fOTUs) in soil. Means within each cropping system are indicated in gray stars.

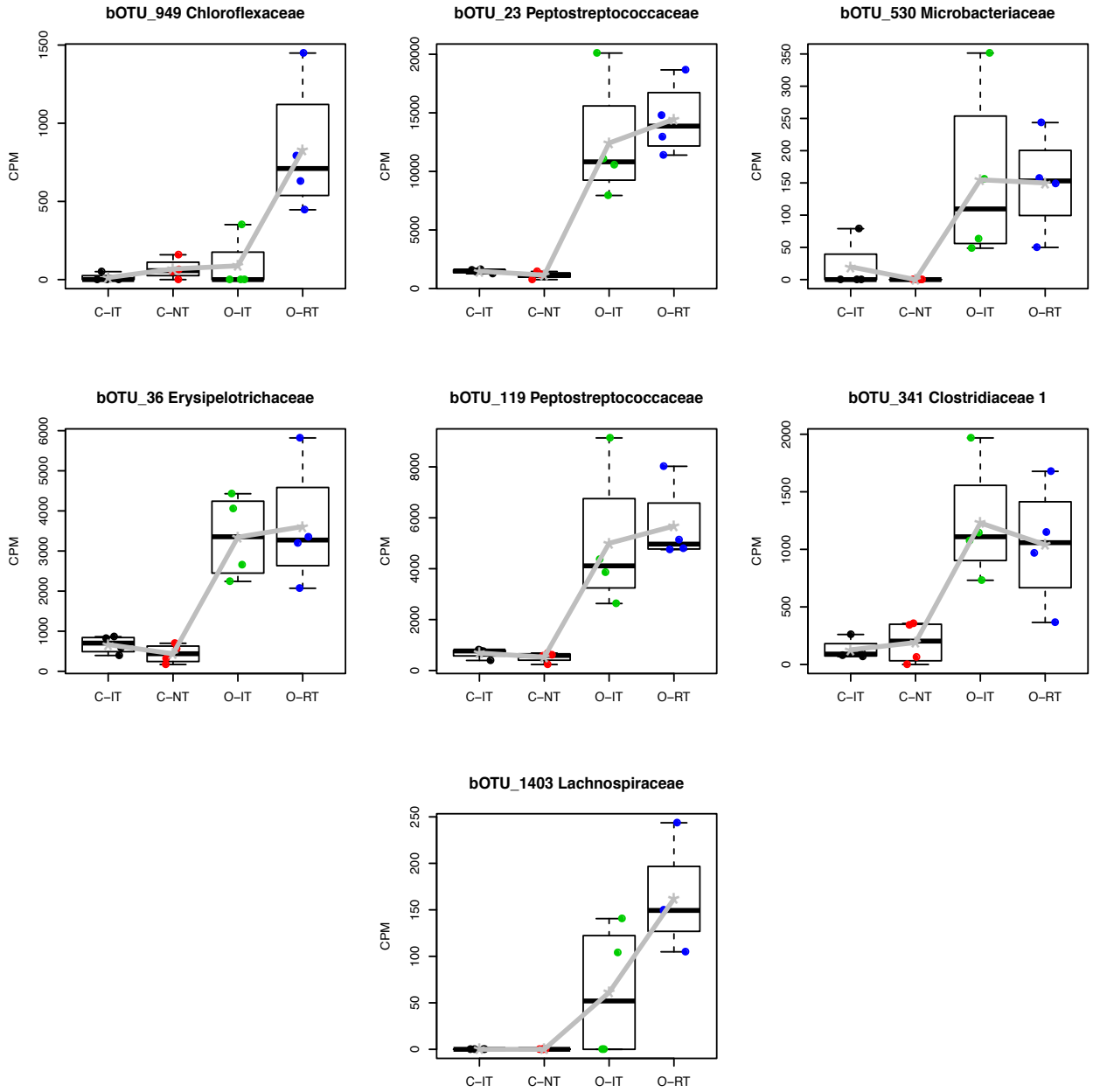

Figure S11: Relative abundances (counts per million, CPM) of abundant cropping sensitive bacteria (bOTUs) in roots. Means within each cropping system are indicated in gray stars.

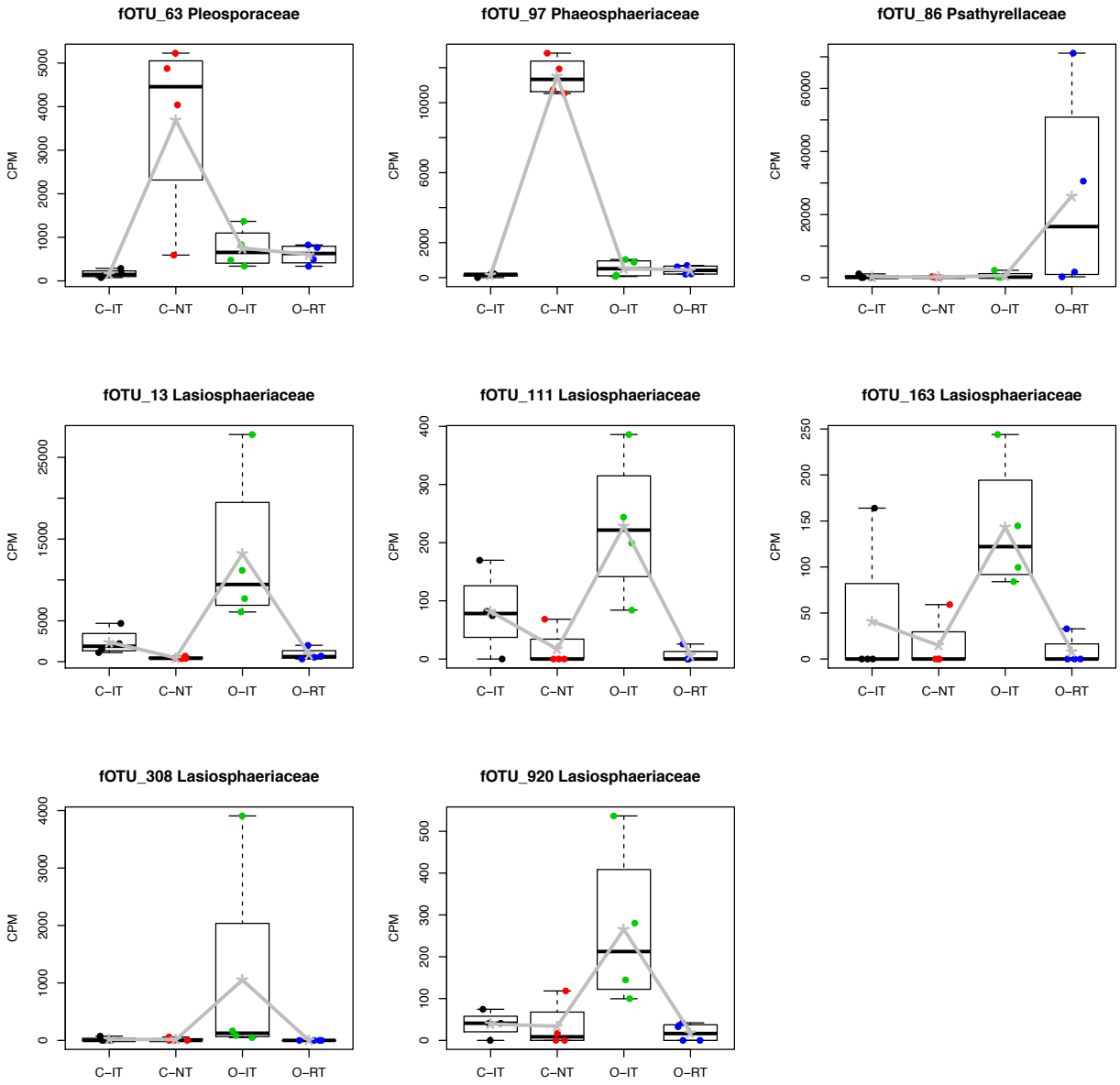

Figure S12: Relative abundances (counts per million, CPM) of abundant cropping sensitive fungi (fOTUs) in roots. Means within each cropping system are indicated in gray stars.

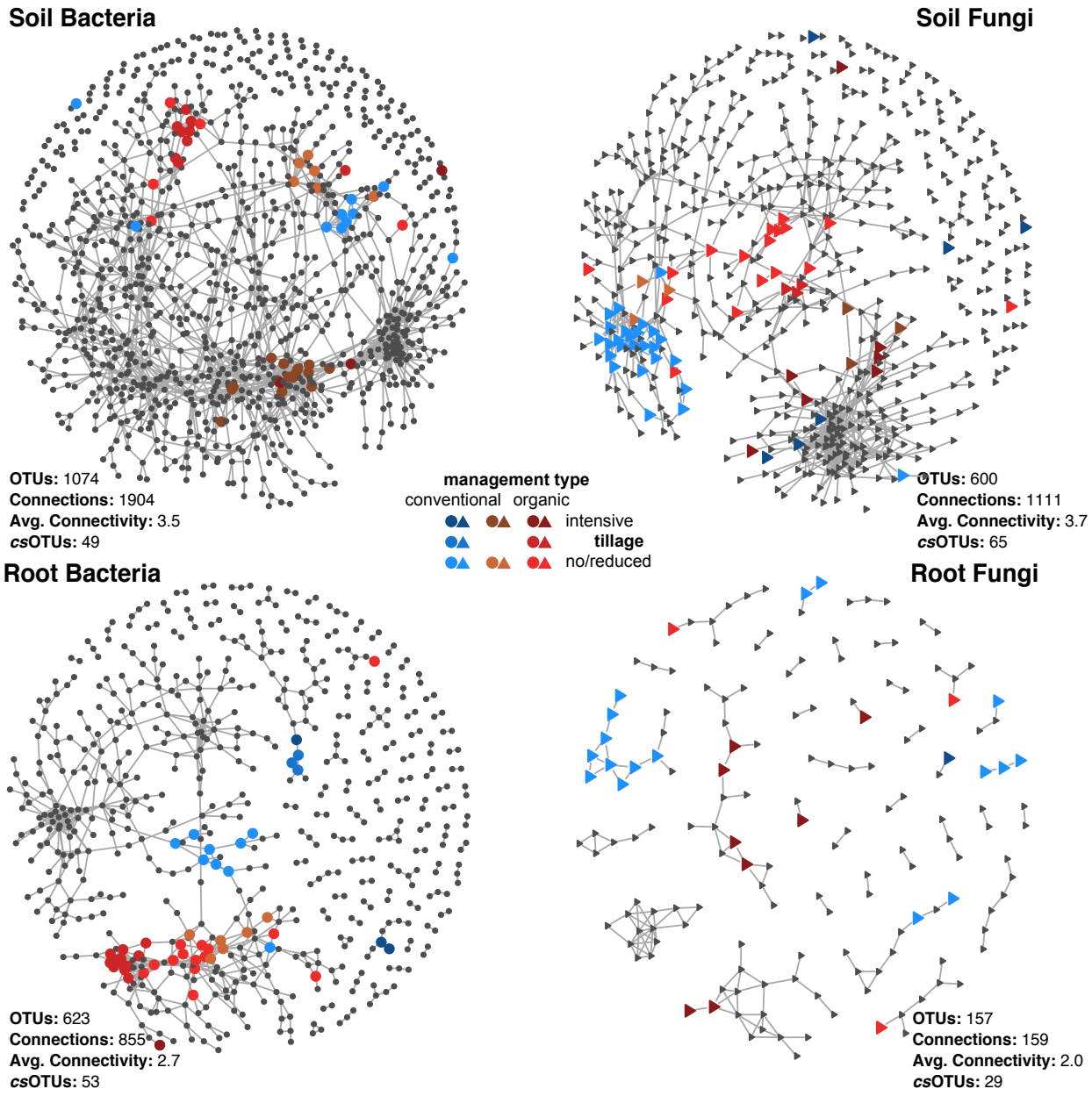

Figure S13: Co-occurrence networks visualizing significant correlations (ρ>0.7, *p<0.001*; indicated with grey lines) between OTU pairs in the soil and root bacterial and fungal communities. Circles and triangles represent bacteria and fungi OTUs, respectively. OTUs were colored by their association to the different cropping systems (as defined in Fig. S6, gray OTUs are insensitive to cropping practices). General network properties are indicated under each network and include: number of OTUs, number of connections, average number of connections between OTUs (avg. connectivity) and the number of *c*ropping *s*ensitive OTUs (*cs*OTUs) in the network.

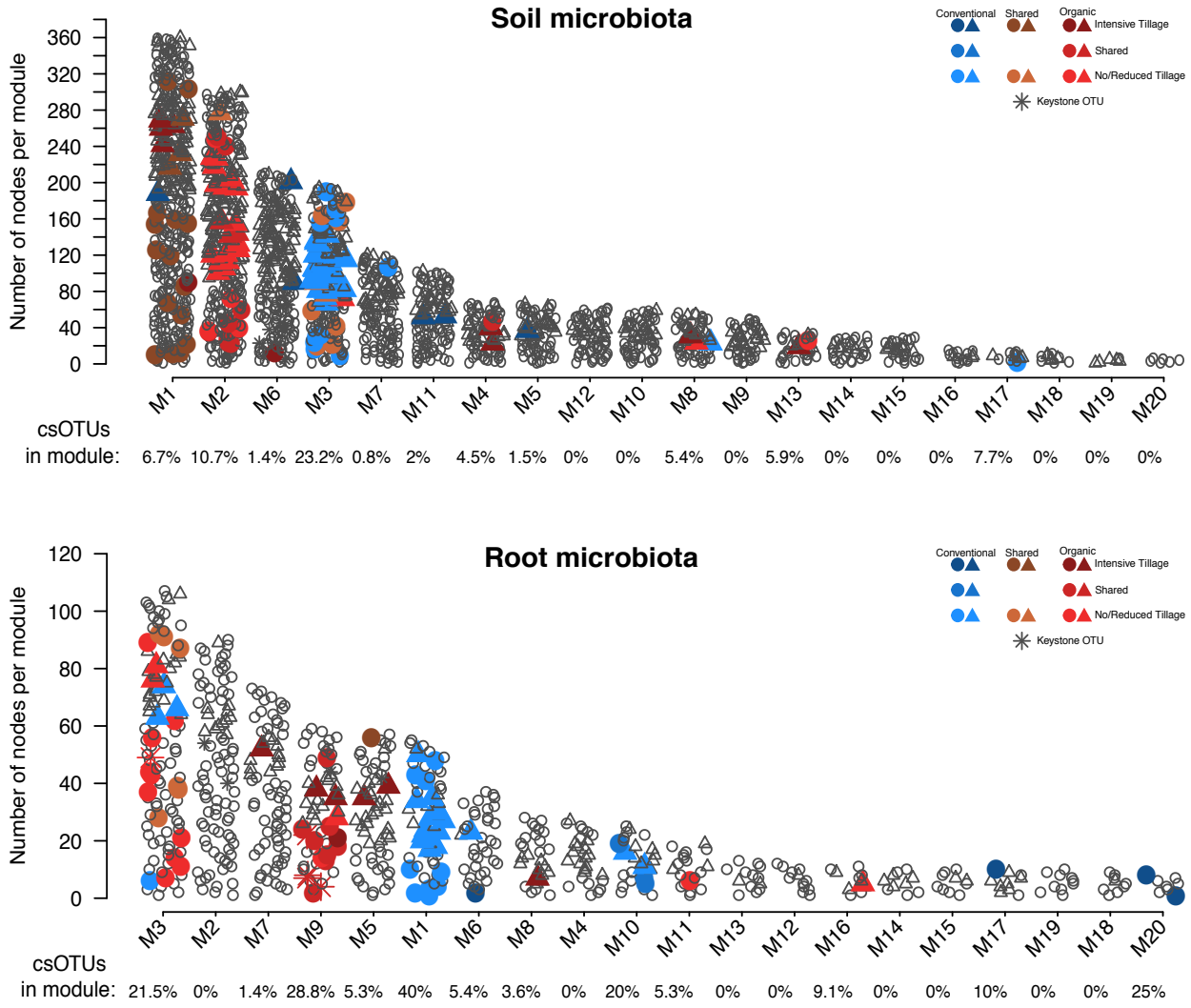

Figure S14: Defining network modules. Plots showing the number of OTUs in the top 20 most populated modules for the soil and root meta co-occurrence networks. Circles and triangles represent bacteria and fungi OTUs, respectively. OTUs were colored by their association to the different cropping systems (as defined in Fig. S6, gray OTUs (open symbols) are insensitive to cropping practices). Percentages on the x-axis indicate the proportion of *cs*OTUs present in each module.

Supplementary Tables

Table S1: PCR cycling conditions used to generate the 16S and ITS amplicons for high-throughput sequencing.

16S				**ITS**			
Step	Temperature	Time	Cycles	Step	Temperature	Time	Cycles
1	94ºC	2min	1x	1	94ºC	5min	1x
2	94ºC	30sec		2	94ºC	30sec	
3	55ºC	30sec	30x	3	57ºC	30sec	30x
4	65ºC	30sec		4	72ºC	30sec	
5	65ºC	10min	1x	5	72ºC	7min	1x
6	15ºC	hold		6	15ºC	hold	

Table S2: Results of PERMANOVA testing the effects of *Block*, *Sample type* and *Cropping System* on bacterial and fungal communities. Significant effects are indicated in bold (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

	Bacteria		**Fungi**	
	pseudo-F	R^2	pseudo-F	R^2
Block $_{(3,21)}$	1.307	0.043	0.922	0.052
Sample type $_{(1,21)}$	**54.665*** **	**0.602**	**19.886*** **	**0.376**
Crop. System $_{(3,21)}$	**2.604***	**0.086**	**1.791***	**0.102**
Type*CropSys $_{(3,21)}$	1.132	0.037	1.285	0.073

Table S3: Statistic testing for differences in α-diversity between root and soil samples in bacterial and fungal communities. Separate t-tests were conducted for each kingdom using a model testing for differences between sample types. Similarly, for each sample type we conducted separate ANOVAs testing the effects of *Block* and *Cropping System*. Significant effects are indicated in bold (**p*<0.05, ****p*<0.001). Different letters in the Tukey pairwise comparisons indicate significant differences at *p<0.05*.

	Bacteria		**Fungi**	
	Soil	**Root**	**Soil**	**Root**
Mean ± SEM	**1058.31 ± 27.44**	**988.31 ± 13.24**	**560.25 ± 10.98**	**239 ± 7.06**
T-test				
Sample type $_{(1,30)}$	**t=2.3***		**t=24.61*****	
ANOVA				
Block $_{(3,9)}$	**F=2.22**	**F=1.91**	**F=1.25**	**F=0.59**
Crop. system $_{(3,9)}$	**F=1.88**	**F=7.77****	**F=1.25**	**F=2.39**
Pairwise Comparisons				
Cropping System	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
C-IT	**1095.25 ± 35.3 a**	**949.25 ± 15.18 a**	**541.25 ± 34.79 a**	**212.25 ± 16.78 a**
C-NT	**1013.25 ± 67.4 a**	**951 ± 30.75 a**	**536 ± 15.19 a**	**252 ± 8.75 a**
O-IT	**1127.75 ± 28.68 a**	**1044 ± 11.51 b**	**583.75 ± 16.91 a**	**256.25 ± 6.84 a**
O-RT	**997 ± 66.8 a**	**1009 ± 7.01 ab**	**577 ± 11.62 a**	**235.5 ± 13.91 a**

Table S4: Results of PERMANOVA testing the effects of *Block* and *Cropping System* on bacterial and fungal communities in soil and root samples. Significant effects are indicated in bold (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Different letters in the pairwise comparisons indicate significant differences at $p<0.05$ (FDR corrected). Results of BETADISP testing for differences in multivariate dispersion between cropping systems in root and soil samples in bacterial and fungal communities.

	Soil				**Root**			
	Bacteria		**Fungi**		**Bacteria**		**Fungi**	
	pseudo-F	R^2	pseudo-F	R^2	pseudo-F	R^2	pseudo-F	R^2
Block $_{(3,9)}$	1.09	0.18	0.73	0.14	1.02	0.17	0.95	0.17
Crop. system $_{(3,9)}$	***1.85******	***0.31***	***1.59****	***0.30***	***2.04*****	***0.34***	***1.54****	***0.28***
Pairwise Cropping System Comparisons								
	C-IT (a) *C-NT (b)* *O-IT (ab)* *O-RT (c)*		*C-IT (ac)* *C-NT (a)* *O-IT (b)* *O-RT (c)*		*C-IT (a)* *C-NT (b)* *O-IT (c)* *O-RT (ac)*		*C-IT (a)* *C-NT (a)* *O-IT (a)* *O-RT (a)*	
Multivariate homogeneity of groups dispersions								
Crop. system $_{(3,12)}$	1.25		1.20		0.61		0.01	

Table S5: Keystone OTUs identified in soil and root microbial communities documented with taxonomy assignments, OTU IDs, node degree values, and cropping system sensitivity.

	Phylum	Class	Order	Family	Genus	Node	Degree	cssOTU
Soil microbial community								
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	unassigned	unassigned	bOTU_537	44	No
	Chloroflexi	Chloroflexia	Chloroflexales	Roseiflexaceae	Roseiflexus	bOTU_443	35	No
	Proteobacteria	Alphaproteobacteria	Rhizobiales	JG34-KF-361	unassigned	bOTU_1110	52	No
				Xanthobacteraceae	Pseudolabrys	bOTU_96	57	No
		Betaproteobacteria	SC-I-84	unassigned	unassigned	bOTU_62	55	No
						bOTU_411	35	No
			TRA3-20			bOTU_180	41	No
		Gammaproteobacteria	Xanthomonadales			bOTU_331	44	No
	Verrucomicrobia	OPB35 soil group	unassigned			bOTU_637	38	No
						bOTU_897	38	No
Fungi	Ascomycota	Sordariomycetes	Chaetosphaeriales	Chaetosphaeriaceae	Chaetosphaeria	fOTU_278	42	No
		Sordariomycetes	Chaetosphaeriales	Chaetosphaeriaceae	unassigned	fOTU_71	42	No
		Sordariomycetes	Sordariales	unassigned		fOTU_641	43	No
	Basidiomycota	Tremellomycetes	unassigned			fOTU_831	57	No
	unassigned	unassigned				fOTU_494	70	No
						fOTU_208	46	No
						fOTU_450	46	No
						fOTU_201	38	No
	Zygomycota	Incertae sedis	Mortierellales	Mortierellaceae	Mortierella	fOTU_337	43	No
Root microbial community								
Bacteria	Acidobacteria	Acidobacteria	Subgroup 2	unassigned	unassigned	bOTU_1141	18	No
	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Leucobacter	bOTU_530	18	**Yes**
		Thermoleophilia	Gaiellales	unassigned	unassigned	bOTU_1091	17	No
	Chloroflexi	Chloroflexia	Chloroflexales	Chloroflexaceae	Chloronema	bOTU_949	17	**Yes**
	Firmicutes	Bacilli	Bacillales	unassigned	unassigned	bOTU_267	21	No
		Clostridia	Clostridiales	Peptostreptococcaceae	Incertae Sedis	bOTU_23	17	**Yes**
						bOTU_119	16	**Yes**
		Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Turicibacter	bOTU_36	16	**Yes**
	Proteobacteria	Alphaproteobacteria	Rhizobiales	unassigned	unassigned	bOTU_54	16	No

Chapter 3:

Deciphering composition and function of the root microbiome of a legume plant

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Abstract

Background: Diverse assemblages of microbes colonize plant roots and collectively function as a microbiome. Earlier work has characterized the root microbiomes of numerous plant species, but little information is available for legumes despite their key role in numerous ecosystems. Legumes form a root nodule symbiosis with nitrogen-fixing *Rhizobia* bacteria and thereby account for large, natural nitrogen inputs into soils. Here we describe the root bacteria microbiome of the legume *Trifolium pratense* combining culture-dependent and independent methods. For a functional understanding of individual microbiome members and their impact on plant growth, we began to inoculate root microbiome members alone or in combination to *Trifolium* roots.

Results: At a whole root scale, *Rhizobia* bacteria accounted for ~70% of the root microbiome. Other enriched members included bacteria from the genera *Pantoea, Sphingomonas, Novosphingobium,* and *Pelomonas*. We built a reference stock of 200 bacteria isolates, and we found that they corresponded to ~20% of the abundant the root microbiome members. We developed a microcosm system to conduct simplified microbiota inoculation experiments with plants. We observed that while an abundant root microbiome member reduced plant growth when inoculated alone, this negative effect was alleviated if this *Flavobacterium* was co-inoculated with other root microbiome members.

Conclusions: The Trifolium root microbiome was dominated by nutrient providing *Rhizobia* bacteria and enriched for bacteria from genera that may provide disease protection. First microbiota inoculation experiments indicated that individual community members can have plant growth compromising activities without being apparently pathogenic, and a more diverse root community can alleviate plant growth compromising activities of its individual members. A trait-based characterization of the reference stock bacteria will permit future microbiota manipulation experiments to decipher overall microbiome functioning and elucidate the biological mechanisms and interactions driving the observed effects. The presented

reductionist experimental approach offers countless opportunities for future systematic and functional examinations of the plant root microbiome.

Keywords: clover, root, microbiome, 16S rRNA sequencing, microcosm

Background

Plant roots in soil are in contact with the most microbially diverse biome on the planet, with estimates of bacteria diversity as high as 38,000 taxa per gram of soil [1]. The root bacteria microbiome typically consists of *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* [2]. Recent studies have highlighted the root bacteria microbiome of several plant species, including *Arabidopsis* [3,4] and a number of crop species, like barley [5], maize [6], sugarcane [7], and rice [8]. However, the microbiome of nitrogen fixing plants, in particular legumes such as red clover, has received little attention in microbiome studies.

Trifolium pratense (red clover, hereafter: Trifolium) is an important forage legume and grown on approximately 4 million hectares worldwide [9]. Because of its beneficial symbiosis with N-fixing rhizobia, Trifolium is cultivated in grass/clover mixtures or as a cover crop in crop rotations [10]. While the species' genetic diversity has been characterized using morphological traits [11], DNA marker polymorphism [12] and genome analyses [13], its root microbiome has not been investigated using high-throughput sequencing tools. Furthermore, Trifolium's association with rhizobia suggests its microbiome may differ from non-legumes in that rhizobia are expected to be highly abundant [14].

The N-provision by rhizobia represents a well-established service to their host. Similarly, other microbiome members were found to assist their host plant in nutrient uptake, protection from pathogens, or modulating immunity responses [15,16]. However, how microbial functions affect plants if a service-providing member is in a diverse community, and how entire microbial communities affect their host, remains poorly understood [16]. One limitation of ribosomal RNA-based root microbiota characterizations is that such approaches only provide indirect information, based upon taxonomic classification, about the function(s) of its members. One suggested approach for the functional examination of the root microbiome relies on isolating root microbes to build microbe collections [17]. The availability of bacterial isolates offers the opportunity for genome sequencing to obtain insights into their potential functions, but more importantly, the activity of these strains can be empirically tested in host-microbiota interaction experiments.

Microbe collections have been assembled [18–22] despite that the recalcitrance to cultivation of many bacteria taxa – with estimates that more than 99% of soil bacteria cannot be cultured [23] – was often seen as a limitation. This recalcitrance does not necessarily apply to bacteria of the root microbiome as evidenced by an earlier study of Chelius and Triplett [24], who reported a phylogenetic overlap of 48% between their bacteria isolate collection and a 16S rRNA clone library from maize roots. More recently, Bai *et al.,* [21] reported a collection of nearly 6,000 root-derived bacteria isolates and a remarkable 54-65% isolation rate compared to the abundant (>0.1% relative abundance) operational taxonomic units (OTUs) in *Arabidopsis thaliana* roots. However, it required considerable effort including large-scale isolation using serial dilutions (seven different bacteria isolation media were used!) and subsequent high-throughput taxonomy identification.

Experimental manipulation of the microbiome and assays with plants require contained systems in which host-microbiota interaction experiments can be conducted without outside microbial contamination. Recently, microcosm systems have been used in combination with bacteria reference stocks to examine the dynamic process of root microbiome assembly from a defined input community under microcosm conditions [21,22]. In these experiments stable and reproducible community assembly was observed. However, these experiments were not designed to clarify how root communities compare to plants grown in artificial substrate in microcosms or in natural soil conditions.

Here we addressed some of the aforementioned research gaps and report a detailed characterization of the Trifolium root bacteria microbiome. We sampled the whole root system including nodules, removed the rhizosphere and investigated the entire root bacterial communities consisting of rhizoplane and endosphere habitats. We utilized a multi-step approach to investigate the composition and culturable fraction of its root microbiome (Figure 1). We also move towards a functional understanding of specific members of the Trifolium root microbiome and developed a microcosm system (Additional file 1: Figure S1a-d) in which we conducted multi-strain inoculation experiments with Trifolium germinated from surface-sterilized seeds and investigated the inoculation-induced effects on plant growth.

Results

Composition of the Trifolium root microbiome

The 16S amplicon sequencing of 24 Trifolium root samples and 15 soil samples from climate chamber and natural site growth experiments (Figure 1, Table 1, Additional file 1: Figure S2,) yielded 9,923,925 high-quality, non-chimeric sequences across all samples, with a

median of 153,072 (range 21,731 – 981,922) sequences per sample (Additional file 2). We rarefied the dataset to an even sequencing depth of 20,000 sequences and identified 3,495 bacteria OTUs and one archaea OTU.

Table 1: Overview of the number of replicate samples by sample type, experiment, and experimental replicate or plot.

	Experimental Soil	Natural Site†			Climate Chamber					
Sample	-	**Plot 1**	**Plot 2**	**Plot 3**	**Ex+ 1**	**Ex 2§**	**Ex 3**	**Ex 4**	**Ex 5§**	**Microcosms**
Root	-	3	3	3	3	3	3	3	3	8◊/12◊◊
Soil	3	3	3	3	3	-	-	-	-	3
Inoculum	-	-	-	-	-	-	-	-	-	4*/3**

† Bacteria isolates from natural site plants were cultured from plants collected from within and outside the experimental plots.
§ Bacteria isolates from climate chamber plants were cultured from these experiments, plus one non-sequenced growth experiment
◊ Total number of samples collected from the soil extract experiment. One root sample was collected from each replicate microcosm
◊◊ Total number of samples from the simplified community experiments. Four root samples were collected from each of the three experiments.
* Independently prepared soil extract samples used as the experimental start inoculum. See Additional file 1 for details.
** One inoculum sample for each microcosm experiment

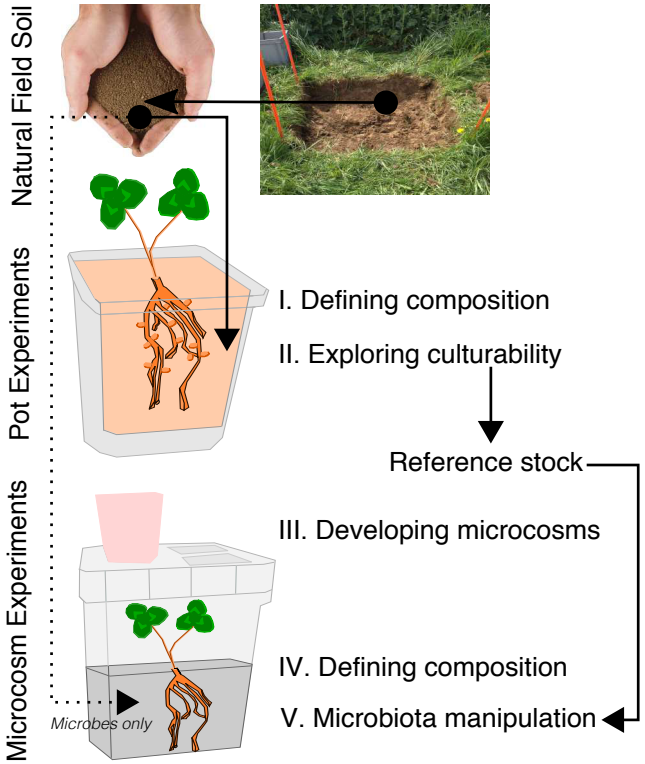

Figure 1: Characterization of the root microbiome. We collected a natural field soil and used it in a series of Trifolium growth experiments. (I) We investigated the composition of the root bacteria microbiome using 16S rRNA sequencing of root samples. (II) We utilized the same root material for an isolation effort to explore the culturable fraction of root bacteria microbiome and assembled a reference stock of bacteria isolates. (III) We subsequently developed a microcosm system to explore plant-microbiota interactions and (IV) investigated the composition of the Trifolium root microbiome in the system by inoculating microbiota extracted from the field soil. (V) We conducted microbiota manipulation experiments in which we inoculated culturable, abundant members of the root microbiome and scored their effects on plant growth.

We confirmed in the Trifolium root microbiome the typical patterns that are often observed in microbial ecology. The soil microbiome is richer and phylogenetically more diverse than the root microbiome (Additional file 1: Figure S3; Table S1). We quantified the major components driving differences between samples (ß-diversity) using unconstrained principal coordinates analysis (PCoA) on weighted UniFrac distances and found a clear separation along Axis 1 (explaining 69.7% of the overall variation) and confirmed the general pattern that soil and roots harbor distinct microbiomes (Figure 2). Axis 2 explained 15.5% of the variation overall and separated mainly the root but not the soil samples, and we did not notice an obvious clustering whether the plants were grown in the same soil in a climate chamber or in the field, suggesting negligible effects of the *growth condition* on β-diversity. We detected a significant effect of *growth condition* on OTU richness only (Additional file 1: Figure S3; Table S1). However, experiment-to-experiment variation (especially climate chamber experiment 2) largely explained the variability between root samples (Additional file 1: Figure S4). Possible effects due to differences in climatic conditions were generally not detected and would have an effect size smaller than replicate experimental variation.

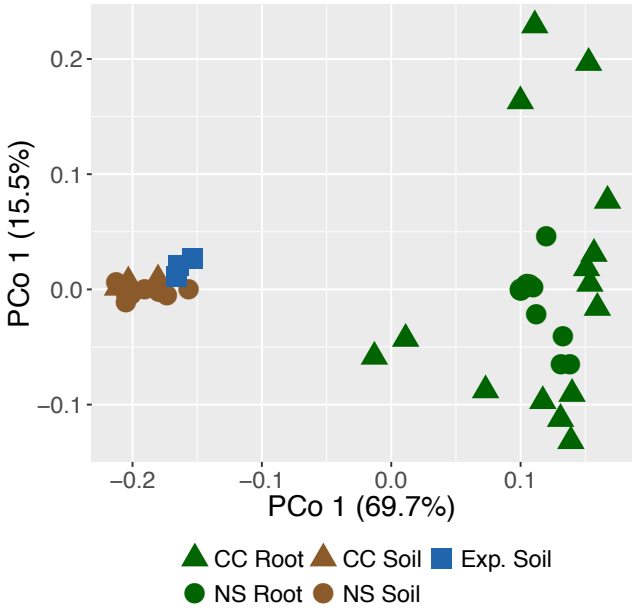

Figure 2: Sample type, growth conditions, and experiment explain much of the variation in soil and root bacteria communities. Unconstrained Principal Coordinates Analysis (PCoA) of weighted UniFrac distances of root and soil samples from climate chamber (CC Root, CC Soil) and natural site growth experiments (NS Root, NS Soil), as well as the unplanted experimental field soil (Exp. Soil). See Additional file 1: Supplementary Figure S4 for points colored by replicate experiment.

In the following, we break down the dissimilarities between soil and root samples to compositional patterns evident in the taxonomic profiles of the samples. Soil samples contained

abundant *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* accounting for a mean of 54.7%, 24.7% and 6.9%, respectively (Additional file 1: Figure S5). The Trifolium root microbiome was dominated by *Proteobacteria* that accounted for a mean abundance of 90.7% across both experimental conditions (Additional file 1: Figure S5).

For the detailed characterization of the Trifolium root microbiome (Figure 1, step I) we first identified the OTUs that were significantly higher in relative abundance in root compared to soil samples and discovered a total of 61 OTUs significantly enriched in root samples (Figure 3), 15 of which were abundant with a mean relative abundance of at least 0.1% across all root samples. These 15 OTUs accounted for 74.5%% of rarefied sequences, and we termed them 'RootOTUs' - referring to the abundant and root-specific members of the Trifolium root microbiome. The RootOTUs consisted mostly of *Proteobacteria* (14 OTUs, Additional file 1: Table S2) and represented six different orders: *Rhizobiales* (6), *Sphingomonadales* (3), *Enterobacteriales* (2), *Burkholderiales* (1), *Caulobacterales* (1), and *Rhodospirillales* (1). The remaining non-*Proteobacteria* RootOTU belonged to the *Firmicutes* and was classified in the genus *Syntrophomonas.* We noted that one RootOTU (*OTU1,* matching *Rhizobium leguminosarum*) dominated the Trifolium root microbiome and explained the high prevalence of *Proteobacteria* (Additional file 1: Figure S5). *OTU1* ranged from 35.4% to 89.7% in samples from both growth conditions and accounted for a median of 73.5% of the root community (Figure 3b). We confirmed that the high abundance of *OTU1* in the overall root community was due to the rhizobia bacteria present in root nodules (Additional file 1: Supplementary methods), and we noted a few non-*OTU1* sequences inside the nodules, suggesting additional within-nodule bacteria diversity (Additional file 1: Supplementary results, Figure S6).

In summary, root bacterial communities did not differ substantially whether the plants were grown under controlled or field conditions, thereby validating our approach using climate chamber experiments. The abundant and root-specific members of the Trifolium root microbiome consisted mainly of *Proteobacteria* and nodule-inhabiting rhizobia bacteria accounted for ~70% of the root microbiome.

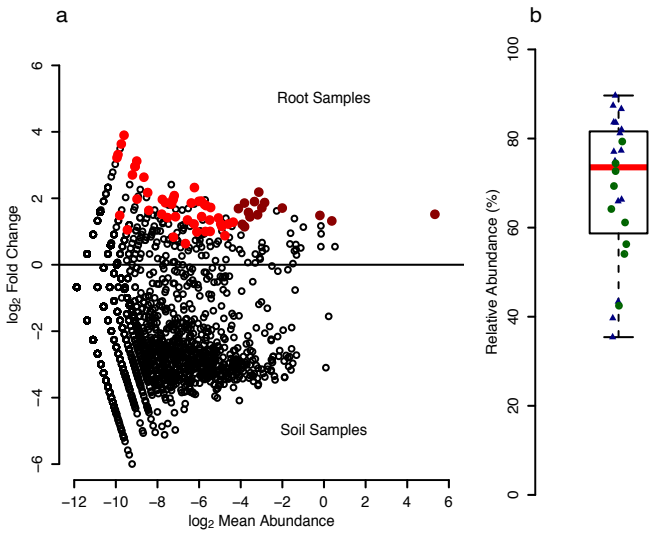

Figure 3: Abundant and root-specific OTUs of the Trifolium root microbiome. (a) The plot reports the mean relative abundance and the $\log_2$ fold change between root and soil samples of all OTUs present in the rarefied community (open black circles). Filled red circles indicate the 61 OTUs significantly enriched ($P < 0.05$, FDR corrected) in root samples. Dark red circles indicate the 15 OTUs present in the RootOTUs (see text). (b) Boxplot (overplotted with individual datapoints) showing the median relative abundance of *OTU1* (*Rhizobium leguminosarum*) in sequenced climate chamber (blue triangles) and natural site (green circles) root samples.

Isolated members of the Trifolium root microbiome

We isolated bacteria from Trifolium roots of two climate chamber experiments and from plants grown at the natural site (Table 1) and characterized a total of 200 cultured bacteria (Figure 1 step II). *Proteobacteria* dominated the culture collection, being represented by 78.5% isolates while *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* accounted for 8%, 8%, and 5.5% of isolates, respectively (Figure 4a). The isolates were assigned to 34 different genera (Figure 4b). The 19 genera of the *Proteobacteria* (157 isolates) included abundant *Pseudomonas* (83 isolates), *Janthinobacterium* (19) and *Stenotrophomonas* (9). We found seven genera in the phylum *Actinobacteria* (16 isolates) with *Microbacterium* (7), *Micrococcus* (3), and *Micromonospora* (2) having more than one representative isolate. In the *Firmicutes* (16 isolates), we noted five different genera, with *Bacillus* (9), *Staphylococcus* (3), and *Paenibacillus* (2) being the most abundant. Finally, we found three genera in the *Bacteroidetes* (11 isolates): *Flavobacterium* (8), *Mucilaginibacter* (2), and *Pedobacter* (1).

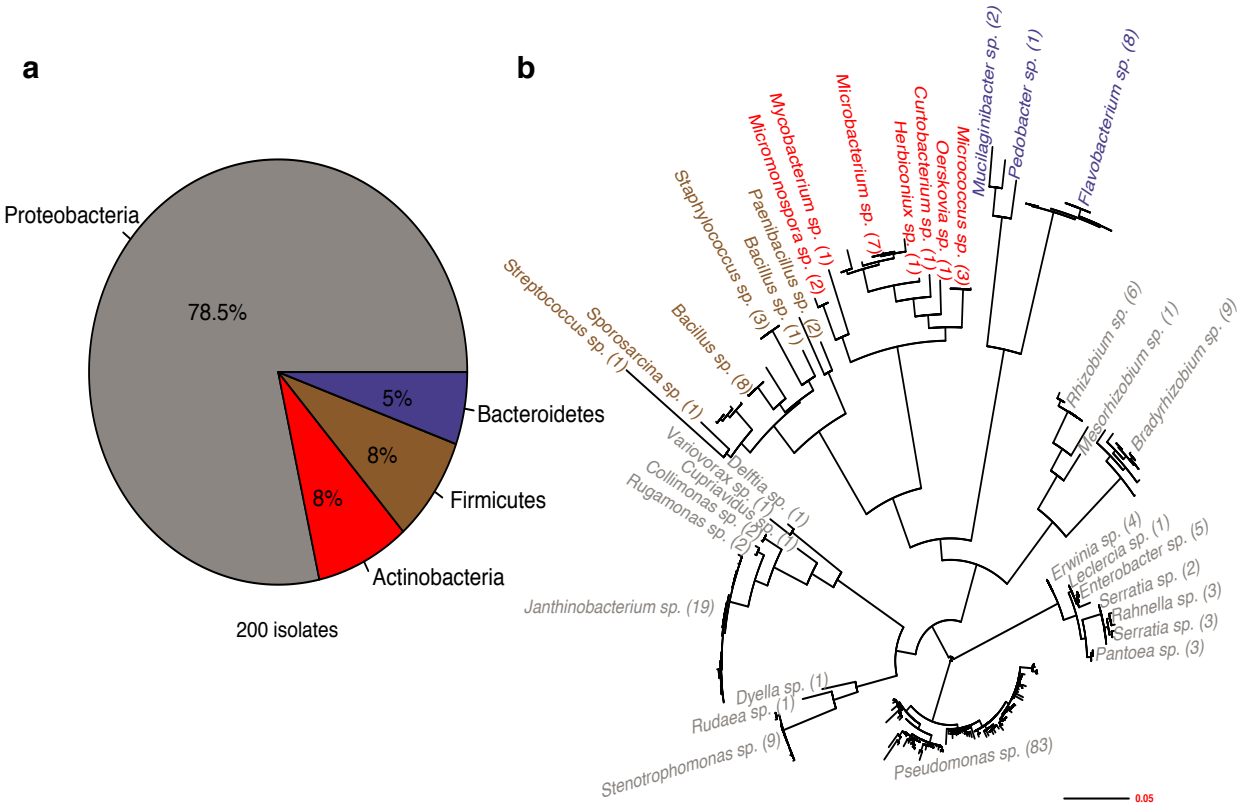

Figure 4: Taxonomic diversity of the Trifolium bacteria reference stock. (a) Taxonomic composition of the isolate collection at Phylum level. (b) The phylogenetic diversity of the isolates at the genus level and the number of isolates assigned to each genus is indicated in parentheses. Isolates are labeled at the genus level and color-coded by phylum in (a).

We clustered the bacteria isolate sequences to the representative sequences of the OTUs of the Trifolium root community profiles at ≥ 97% sequence similarity (see Supplementary methods) and determined whether a bacteria isolate constituted an abundant and root-enriched member of the Trifolium microbiome. Overall, out of the 200 bacteria isolates, 181 (90.5%) isolates clustered to 34 OTUs of the root community profile while for 19 (8.5%) isolates we did not find a matching community member. All of the 34 isolated OTUs were present in the rarefied root community (2,426 OTUs), corresponding to an isolation rate of 1.4% (Figure 5). The isolation rate increased to 23.6% when comparing to the abundant community members: 55 abundant OTUs had a mean relative abundance of ≥ 0.1% across all root samples, and for 13 of these, we were able to culture bacteria strains. We identified 11 bacteria isolates for 2 of the 15 RootOTUs (Figure 3; Additional file 1: Table S2). The cultured RootOTUs included the dominant *OTU1* (*Rhizobium leguminosarum*; 5 isolates), as well as *OTU48* (*Pantoea agglomerans*; 6 isolates).

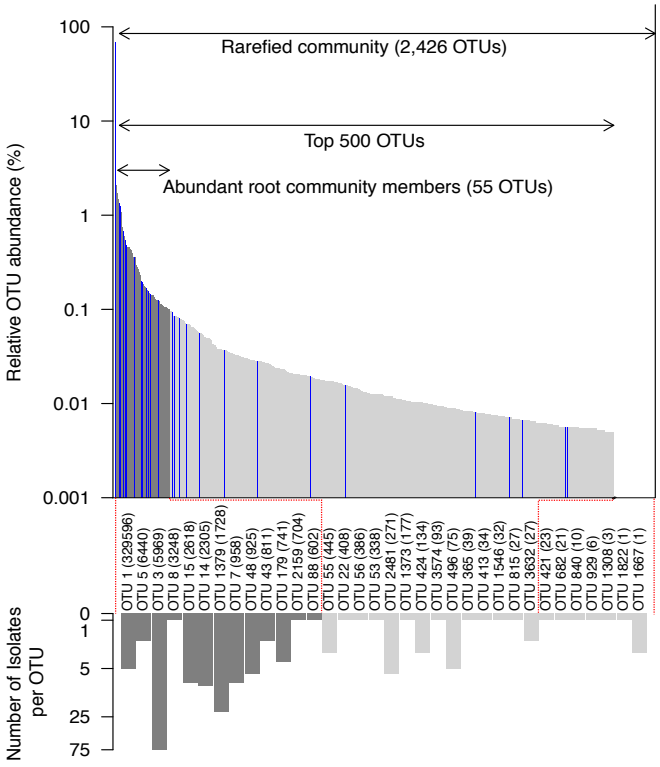

Figure 5: Mapping of reference stock bacteria to root microbiome OTUs. The upper bar graph represents the relative abundance of the 2,426 OTUs in the root-associated bacteria community of Trifolium, with the 500 most abundant OTUs shown in gray bars. The dark gray bars indicate the 55 most abundant root OTUs (mean RA >0.1%). Blue bars indicate OTUs for which at least one isolate is present in the reference stock. The lower, inverted bar graph indicates the number of isolates in the reference stock mapping to an OTU in the community profile. Bars are shaded the same as in the upper graph to indicate the relative abundance of each OTU. Bars are labeled with the representative OTU name and its total number of sequences in the community profile in parentheses.

We concluded that almost a quarter of the abundant root community members can be obtained in culture, and we achieved this with a manageable effort (200 strains) and straightforward microbiological techniques. By linking to the information of the root community profiles we have characterized the bacteria strains of the reference stock with rank and relative abundance in the Trifolium root microbiome, and thereby the reference stock represents a toolbox for future microbiota manipulation experiments.

Towards functional investigations of the Trifolium root microbiota

Finally, we developed microcosms (Figure 1, step III) and evaluated their potential to conduct plant-microbiota interaction experiments. Recent microbiota inoculation experiments [21,22] revealed that approximately half of the inoculated bacteria strains previously isolated from roots of soil-grown *Arabidopsis* either completely failed or failed to robustly colonize the roots of their host plant under microcosm conditions. We speculate that this could partly be due to the different physical and chemical conditions in the microcosms compared to soil, and

that these conditions are unfavorable for certain isolates. Therefore, we performed a soil extract experiment to pre-screen for possible microcosm-adapted bacteria strains. For this, we characterized the root microbiome of Trifolium that assembled after inoculation of a diverse soil microbiota extracted from the experimental field soil (Additional file 1: Figure S7a,b, Figure S8; Supplementary methods and results). We defined the root bacteria community (Figure 1, step IV) and determined which bacteria isolates (from the reference stock, Figure 5) corresponded to abundant OTUs on the roots under microcosm conditions (Additional file 1: Figure S8; Supplementary methods). See the supplementary results for a comparison between microcosm and soil-grown root communities (Additional file 1: Figure S9a,b; Supplementary methods and results).

We then conducted microcosm experiments in which we inoculated Trifolium in the microcosms with bacteria strains isolated from its root microbiome. The goal was not to screen strains or to test specific functions, but instead to combine all our tools (reference stock, microcosms, community sequencing, and soil extract information) and validate the overall experimental approach for future microbiota inoculation experiments. We assembled a simplified community, choosing strains from the reference stock that corresponded to abundant OTUs on the roots under microcosm conditions *and* belonged to well-represented bacterial genera in the collection (Figure S9; strains per OTU were randomly chosen): a *Flavobacterium* (*F*; *Bacteroidetes*, #8 isolates for this genus in the reference stock; KHB002), a *Pseudomonas* (*P; Proteobacteria,* #83; KHB004) and a *Janthinobacterium* (*J*; *Proteobacteria,* #19, KHB023; Table 2). We also included a *Microbacterium* (*M*; *Actinobacteria*, #7; strain KHB073) because this genus was well-represented in the reference stock (numerous isolates could indicate that these bacteria were abundant on roots; Figure 4b) and because we wanted the inoculated community to broadly reflect the abundant bacterial phyla of plant root microbiomes (*Actinobacteria*, *Bacteroidetes* and *Proteobacteria*; [2,25]). We inoculated these bacteria alone or in combination to the autoclaved microcosms (Figure. 1, step V) at densities of 10^6 cells ml^{-1} and planted surface sterilized Trifolium seeds. We then monitored the community dynamics of the inoculated simplified community and scored effects of the bacteria inoculation on plant growth in three replicate experiments.

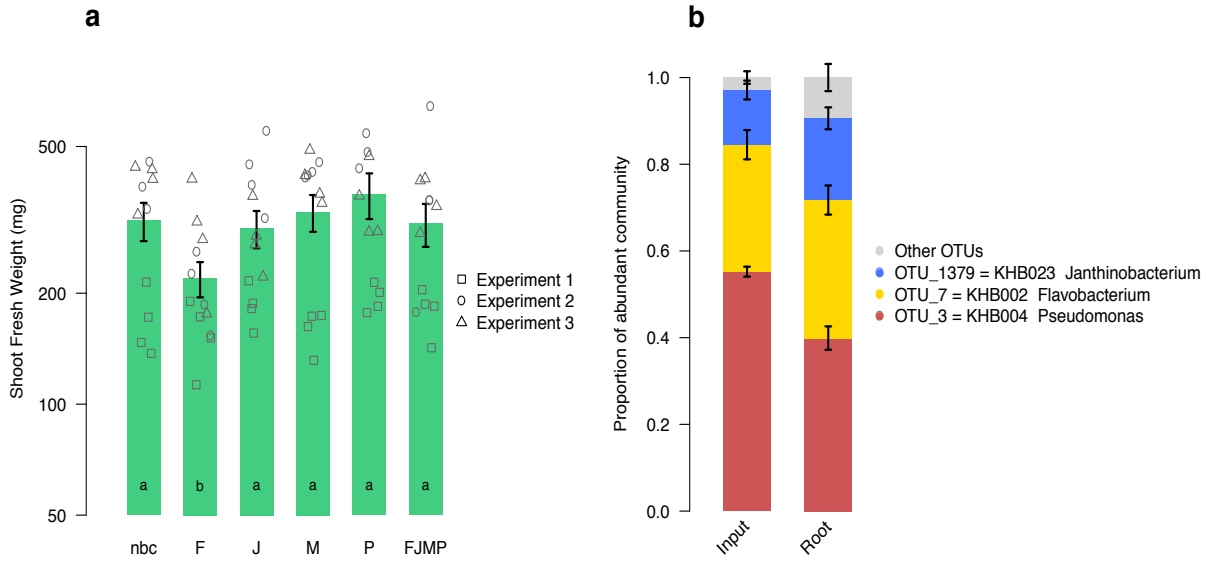

Figure 6: Functional analysis of a simplified Trifolium root microbiota in microcosms. (a) Trifolium growth in microcosms in absence of inoculated bacteria (nbc: no-bacteria control), with specific strains (F: Flavobacterium KHB002; J: Janthinobacterium KHB023; M: Microbacterium strain KHB073; P: Pseudomonas KHB004); or the simplified community (FJMP). The graph reports the mean shoot fresh weight (n=12; ± s.e.m) and the individual data points from the three independent experiments with 4 replicates each. Letters indicate statistical significance at $P < 0.05$ (Tukey's HSD; analysis over the three experiments). Note, the Microbacterium (M, panel **a**) was not captured with the community quantification method. (b) Community composition of the simplified community (FJMP) at inoculation (input) and after 25 days on the roots. Sequences of other OTUs are indicated in gray.

After 25 days, we harvested the experiments and counted $\geq 10^6$ bacterial colony forming units of the inoculated strains on the roots (Table 2). This confirmed that the chosen strains are also able to successfully colonize roots under the artificial growth conditions in the microcosms. We noted a lower biomass in one experiment compared to the two others, and this experiment-to-experiment variation indicated to us that numerous replicates are needed also when highly controlled conditions are used. With regard to the effects of individual bacteria inoculation on the plants, we found that the *Flavobacterium* negatively affected the growth of Trifolium, while the other bacteria did not have an effect on shoot biomass production (Figure 6a). The combined application of the bacteria (*FJMP*) also did not have an apparent effect on biomass production but alleviated the negative impact of the *Flavobacterium* when grown alone. We measured the composition of the simplified community upon inoculation and after 25 days on the roots (Additional file 1: Supplementary methods for details). The *Microbacterium* could not be captured with the community quantification method, and we noted a small proportion of additional OTU sequences possibly representing sequencing errors or contamination, or in root samples, being derived from seed endophytes. Despite these limitations, the analysis revealed that the three other inoculated members retained similar proportions on the roots during 25 days of incubation as compared to when they were

inoculated (Figure 6b). This observation indicated that the alleviation of the negative impact of the *Flavobacterium* was not due to out-competition of this community member, but rather that its negative activities may have been "buffered" by the other bacteria in the simplified community.

Table 2: Bacterial strains used in the microcosm experiments

StrainID	Phylum	Genus	Species°	Abb.§	OTU	Colonization†
Control	-	-	-	NBC	-	$> 1 * 10^{2\Delta}$
KHB073	Actinobacteria	Microbacterium	*M. sp.* or *oxydans*	M	n.d.*	$7.80 * 10^{6}$
KHB002	Bacteroidetes	Flavobacterium	*F. succinicans*	F	OTU_7	$3.51 * 10^{6}$
KHB004	Proteobacteria	Pseudomonas	*P. veronii* or *fluorescens*	P	OTU_3	$5.48 * 10^{7}$
KHB023	Proteobacteria	Janthinobacterium	*J. lividum*	J	OTU_1379	$2.95 * 10^{7}$

° Taxonomy based on Greengenes 16S database [51]
§ Abbreviation
† Mean bacterial cell number on roots after 25 days in the microcosms in Experiment 3.
Δ Highest order of magnitude at which observed OTUs were recorded
* Not detected in the Trifolium root microbiome using high throughput sequencing

Discussion

Root microbiome composition

Here, we have characterized the bacterial communities on roots of *Trifolium pratense* with respect to their composition and reported first steps towards experimentally testing their functions. Trifolium harbors a diverse root microbiome that differs qualitatively and quantitatively from that of the surrounding bulk soil (Figure 2), confirming studies with other plant species [3–5,26]. We found that *OTU1*, matching *Rhizobium leguminosarum*, accounted for a median 73.5% of the root microbiome (Figure 3b). We separately inspected root nodules and confirmed that Trifolium nodules were primarily inhabited by *R. leguminosarum* (Additional file 1: Figure S6) but also contained other bacteria taxa. This is in agreement with earlier work revealing within-nodule diversity in *T. repens and T. fragiferum,* which consisted of the dominant *R. leguminosarum* and the less-frequent rhizobia species *Bradyrhizobium japonicum*, *Sinorhizobium sp*. and *Mesorhizobium* [27,28]. For the purpose of the microcosm experiments we described the root microbiome of Trifolium at a whole-root scale, sampling the entire root system including nodules. For a broader description of legume microbiomes, future work investigating the variation in multiple soil types and comparisons with non-legume plants are needed. Additionally, an in-depth spatial assessment of legume root microbiomes would be insightful, e.g. by profiling the bacteria communities of root tissues with the nodules removed as well as inside the root nodules.

The large number of DNA sequences allowed us to thoroughly characterize the Trifolium root microbiome beyond the dominant rhizobia members. In addition to *Rhizobium*, Trifolium supports enriched OTUs from the genera *Pantoea, Sphingomonas, Novosphingobium,* and *Pelomonas,* among others, in its root microbiome (Additional file 1: Table S2). A review of relevant literature reveals that bacteria isolates of some of these genera have been found to be antagonistic to pathogens (Additional file 1: Table S2). This could possibly suggest a partitioning of complementary host services in the Trifolium root microbiome with 'disease protection' and 'nutrient provision' provided by the mentioned root-enriched genera and the nodule-inhabiting *Rhizobia*, respectively. However, because it is notoriously problematic to infer bacteria function from a taxonomy assignment [29], approaches other than 16S community sequencing are required for the functional understanding of the root microbiome. As a next step, such an indicative observation from cultivation independent microbiome analysis could be examined by testing reference stock bacteria belonging to these OTUs for their ability to suppress pathogens.

Reference stocks and microcosms to study functions of the root microbiome

With the isolation of root microbiome members (Figure 5), setting up an experimental microcosm system (Additional file 1: Figure S1a-d) and testing for microbiota effects on plant growth (Figure 6a), we delineate a possible approach to advance the functional understanding of the root microbiome. We built our reference stock (Figure 4b) using one bacteria isolation medium, and at a sampling depth of 200 bacteria strains we captured close to a quarter of the abundant members of the Trifolium root microbiome. Therefore, we believe that our work presents an encouraging example especially for smaller laboratories with limited resources. For future work, additional isolation media and growth conditions would likely permit us to broaden the reference stock and contribute to a targeted cultivation of "missing" Trifolium root microbiome members.

Experimentation with inoculated plants

We conducted multi-strain inoculation experiments with members of the Trifolium root microbiome to evaluate the suitability of microcosm growth system for plant-microbiota inoculation experiments. However, we first conducted the soil extract experiment (Additional file 1: Figure S7a,b, Figure S8; Supplementary methods and results) as a proof-of-concept to pre-screen microcosm-adapted bacteria strains. We subsequently tested four bacteria strains, three of which were culturable members of the abundant root community (Figure 5) and were also abundant members of the root microbiome in the soil extract experiment (Additional file

1: Figure S8). We chose to include a *Microbacterium* isolate because of its abundance in our reference stock (7 isolates, Figure 4b) and its classification in the *Actinobacteria*, a phylum shown to be abundant in plant root microbiomes [25]. We confirmed that these strains successfully colonized plant roots as suggested by the higher abundances on roots compared to their initial inoculated density to the microcosms (Table 2).

We could not capture the *Microbacterium* strain with the community quantification method (Figure 6b), and similarly, none of the seven isolates from the reference stock clustered to any OTU in the entire dataset. A first possible explanation is that the *Microbacterium* is a rare but easily culturable microbiome member. Alternatively, the *Microbacterium* could be an abundant microbiome member, as indicated by the numerous isolates in the reference stock, but absent in the community profiles because of an observed mismatch in the priming site of the PCR primer 799F. A third possible explanation for the microcosms is that although the titer quantification revealed that the *Microbacterium* strain successfully colonized the plant roots in mono-associations, this strain was out-competed in the simplified community by the other tested strains. Future experiments need to clarify among these possibilities, but nevertheless, this is an example where cultivation and DNA-based approaches do not overlap, and a reminder that both methods have inherent limitations. While it is often discussed that PCR primers are biased towards certain bacterial taxa [30], the same is also true for isolation media, which have a specificity by favoring growth of certain bacterial groups [31].

We quantified the fresh weight of the shoot biomass in response to the bacteria in mono-associations or when the four bacteria were combined to a simplified community. We found that plants grew smaller when inoculated with the *Flavobacterium* strain in a mono-association, but that this negative plant growth response was alleviated when the *Flavobacterium* was inoculated in a community with the other strains (Figure 6a). Since we measured that the *Flavobacterium* comprised roughly a third of the community (Figure 6b), we excluded the possibility that the loss of the negative growth effect was due to the bacterium being outcompeted by the other inoculated strains. Instead, the growth compromising activities of the *Flavobacterium* were possibly counteracted by one or more of the co-inoculated isolates, or alternatively, it did not reach a sufficient cell density in the simplified community treatment.

The reference stock bacteria and microcosms present valuable resources for future microbiota manipulation experiments in which the contribution of the plant root microbiome to plant growth can be investigated. One next step would be to identify the functional traits, e.g. related to bio-control or plant-growth promotion, of the reference stock bacteria using bioassays and/or genome sequencing. We expect that different strains that mapping to the same

OTU would interact differently with the host plant, and thus the testing of the functional range among bacteria within an OTU presents another next step. In summary, there are countless opportunities for microcosm experiments. For example, the microbiota of Trifolium can be manipulated with regard to its taxonomic or trait composition or with regard to its diversity and tested for effects on plant growth. Furthermore, the interplay among community members or the dynamics of community assembly can be examined in more detail. Finally, microbiota induced effects on plant growth under stress conditions such as high salinity, reduced nutrient availability, or pathogens can be investigated.

Conclusion

We have reported a multi-step approach (Figure 1) combining cultivation dependent and independent methods to describe and functionally examine the root microbiome of Trifolium. The need to experimentally manipulate a microbiota requires reference stocks of isolates, and we believe that reductionist plant-microbiota systems will permit a systematic examination of the root microbiome functions. Further studies employing targeted manipulations of the root microbiome can help in the development of new tools to increase the sustainability of other agricultural plant species [17] and investigate the relationship between microbiome diversity and plant performance [16].

Methods

Preparation of experimental soil, plant cultivation and harvest

Experimental soil: All experiments of this study were conducted with a natural experimental soil collected from the area outside the experimental plots of the long-term Farming Systems and Tillage (FAST) experiment (47°26'20" N 8°31'40" E). The experimental soil is a loamy sand with the following physicochemical characteristics: pH 6.11; 16/31/51 % clay/silt/sand; 19.37/1.25/4.88 mg/kg N/P/K (measured in 1:10 water extract by Eric Schweizer AG, Thun, Switzerland). In March 2013, we manually excavated three 1 m^2 plots to a depth of 30 cm. The top layer of vegetation (5 cm) was removed and the remaining bulk soil was collected, passed through a 2 mm sieve, homogenized and stored at 4°C until use.

Plants: Seeds of *Trifolium pratense* var. Milvus were surface-sterilized (10 min. in 70% ethanol, then 10 min. in 5% bleach and two washes with sterile H_2O) and cultivated under controlled conditions (16h/25ºC days, 8h/16ºC nights; Additional file 1: Table S3) in climate chambers (Sanyo MLR-352H; Panasonic, Osaka, Japan) and natural conditions in a field experiment. For the climate chamber experiments, pots (8 x 8 x 8.5 cm) were filled with

experimental soil, 15-20 sterilized seeds were sown in the center each pot and after 1 week of growth, the germinated seedlings were thinned until 1 plant per pot remained. The plants were watered 2-3 times per week with distilled H_2O. We conducted five independent replicate climate chamber growth experiments (Additional file 1: Figure S2). We also conducted a field experiment in April 2013 using the three excavated plots from the soil collection effort (see above). A polycarbonate plastic ring (⌀ 30 cm, height 20 cm) was placed in the center of each plot and filled with the experimental soil (homogenized, sieved to 2 mm). The remaining area outside the plastic ring was filled with regular field soil. A few sterilized seeds were sown in each plot and covered with a thin layer of experimental soil (Additional file 1: Figure S2). During the growth period, the plots were weeded twice but otherwise exposed to natural conditions and not managed.

Harvest: The climate chamber plants were harvested after nine weeks and the field experiment was harvested once the plants reached the same growth stage as the plants in the climate chamber (14 weeks, Additional file 1: Figure S2). The entire soil volume inside the plastic ring with the aboveground plants was harvested and brought to the laboratory where the plants were processed. The roots were shaken to remove bulk soil, rinsed with distilled H_2O to remove the rhizosphere (adhering soil particles), and we then sampled the 5 cm fragment of the root system corresponding to the soil depth between -1 and -6 cm using a scalpel in a Petri dish. The 5 cm root fragment presented the same sampling unit used for DNA-extraction and for isolation of bacteria. Because our sampling method does not discriminate between microbes inhabiting the inner root tissue, root nodules, or the root surface, we refer to the profiled community as "root"-associated' or simply, "root" microbiome and do not differentiate between the different compartments. We also collected soil aliquots of the climate chamber and plots of the field experiment by sampling plant root-free bulk soil into 2 mL plastic tubes. The soil samples were flash-frozen in liquid nitrogen and stored at -20ºC until further processing.

16S rRNA community profiling

Detailed information regarding the sequencing approach is available in Additional file 1: Supplementary methods.

DNA extraction: Three 5 cm root fragments were combined into a 15 ml plastic tube making up one DNA sample, and we prepared three replicate DNA samples per experiment (9 root samples total). Similarly, for the field experiment, nine plants per plot were sampled and divided equally to make three replicate samples per plot. DNA was extracted using the

FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions (Additional file 1: Supplementary methods for further details).

PCR, library preparation and sequencing: We used the primers 799F [24] and 1193R [32] flanking the variable regions V5-V7 of the 16S rRNA gene [33]. The 5' end of the forward primer was amended with a unique 6-mer barcode selected from Faircloth and Glenn [34] (Additional file 2). See Additional file 1: Supplementary methods for details related to PCR and purification. Library preparation and sequencing were conducted at the Functional Genomics Centre Zurich (http://www.fgcz.ch) on the Illumina MiSeq Personal Sequencer (Illumina, San Diego, CA, USA).

Sequence processing: The raw reads were processed using an in-house-developed bioinformatics pipeline, which is available in Additional file 3. Briefly, the raw paired-end reads were quality filtered and trimmed at the 3'-end to 280 bp using PRINSEQ v0.20.4 [35] to improve the merging success and reduce error rate [36]. The trimmed paired-end reads were merged with FLASH v.1.2.9 [37]. Sequences from individual samples were de-multiplexed according to the forward barcode using Cutadapt v1.4.2 [38]. The merged 16S sequences were quality filtered with PRINSEQ and for OTU delineation truncated at a fixed length of 360 bp, sorted by abundance, de-replicated, and clustered to operational taxonomic units (OTU, ≥97% sequence similarity, singletons removed) with UPARSE v8.0.1623 [39]. Amplicons were chimera-screened against the GOLD database v.5 [40] and removed. Taxonomy assignment of the OTU representative sequences was performed using the SILVA 16S v119 database [41] with the RDP classifier as implemented in QIIME v1.8 [42].

Statistical analysis of community profiles

All analyses were performed using R v3.1.2 [43] and different R packages. The R code and input files required to replicate all analyses and figures is available in Additional file 4, and the approach is outlined in Additional file 1: Supplementary methods. Briefly, the OTU and taxonomy tables were filtered to exclude OTUs classified as Eukaryotes, chloroplasts, and mitochondria. The OTU table was rarefied to 20,000 sequences per sample (Additional file 1: Supplementary methods, Figure S10), and the abundance of each OTU was expressed as percentages of the total number of counts in a sample. All statistical analyses were performed on $\log_2$+1 transformed data. All *P*-values were adjusted for multiple comparisons with the false discovery rate (FDR) correction using the Benjamini-Hochberg method [44]. We made use of the R-packages *vegan* v2.3-5 [45], *picante* v1.6-2 [46] and the Bioconductor package *phyloseq* v1.14 [47].

Bacteria reference stock

Detailed information regarding isolation, sequencing, and taxonomic assignment of bacteria isolates is available in Additional file 1: Supplementary methods.

We isolated root-associated bacteria from two climate chamber experiments and from Trifolium individuals collected from the field site by plating serial dilutions of a root slurry onto Flour medium agar [48] plates amended with 10 μg mL^{-1} Cycloheximide (to inhibit fungal growth; Sigma Aldrich, St. Louis, MO USA). DNA extracted from single colony isolates was subjected to PCR using the primers 27F [49] and 1401R [50] and Sanger sequenced with 1401R as the sequencing primer by Microsynth AG (Balgach, Switzerland). These sequences were used for taxonomy assignment using the RDP classifier against the SILVA (v119) [41] database as implemented in QIIME [42]. 23 isolates could not be assigned using SILVA and were further classified against the 16S ribosomal RNA database using NCBI BLAST. Additional file 5 gives the unique ID, source of isolation, taxonomy information, and 16S rRNA sequence and for each isolate.

Microcosm experiments

Detailed information regarding the design of the microcosms and bacteria community experiments is available in Additional file 1: Supplementary methods.

We constructed experimental microcosms from Magenta GA-7 boxes (Sigma Aldrich, St. Louis, MO USA) and filled them with 70g of a calcined clay marketed as OilDri (Damolin GmbH, Oberhausen, Germany) (Additional file 1: Supplementary methods, Figure S1a,b). Microcosms containing the artificial soil substitute were covered with aluminum foil and sterilized by autoclaving (2x 99 min at 121˚C). We pre-germinated surface-sterilized Trifolium seeds (see above) for four days under controlled conditions in a climate chamber (Additional file 1: Table S3) on square Petri dishes containing 0.5x Murashige and Skoog basal medium (Sigma Aldrich, St. Louis, MO USA) supplemented with 1% sucrose. Seedlings with roots of ~1 cm length that were free of visible contaminations, but potentially containing endophytes, were used to conduct a microcosm experiment to assess the effect of four bacteria strains, inoculated individually and in combination, on plant growth (Additional file 1: Figure S1c-d). We determined the community profiles of the start inoculum of the combination treatment samples (3 independent preparations) and the root samples using the 16S rRNA sequencing approach described above. The sequences of samples from all microcosm experiments were co-clustered with the sequences of the field- and climate chamber grown Trifolium for community comparisons across experiments. We subsequently assessed the effect of the

bacteria treatments on plant shoot biomass in the three replicate experiments using Two-way analysis of variance (ANOVA). Significant differences between the different treatments were assessed with Tukey's Honest Significant Differences (HSD) test and were considered significant at $P < 0.05$.

Additional Files

Additional file 1: Supplementary methods. Expanded description of all experimental methods. **Supplementary results.** Results of the clone library analysis and soil-extract microcosm experiment. **Supplementary discussion.** Discussion of root microbiome assembly in microcosms. **Figure S1.** Photos documenting the setup and planting of the microcosm experiments. **Figure S2.** Time-course photos of climate chamber and natural site Trifolium growth experiments. **Figure S3.** Rarefaction curves and α-diversity of root and soil samples in both growth conditions. **Figure S4.** PCoA plot colored individually by replicate Trifolium growth experiment. See Figure 2 in main text. **Figure S5.** Weighted UniFrac clustering of Trifolium root and soil samples linked to differences in phyla abundances. **Figure S6.** Clone library sequences clustering to OTUs from the root community profiles. **Figure S7.** Quantitative and qualitative comparisons of soil extract inoculum α-diversity to native field soil. **Figure S8.** Abundant root OTUs in the soil-extract microcosm experiment. **Figure S9.** PCoA plot of inoculum, substrate, and root samples from the soil-extract microcosm experiment clustered with native field soil and climate chamber root samples and a comparison between microcosm and climate chamber root communities. **Figure S10.** Box plot of sequencing depth across climate chamber and natural site root and soil samples. **Table S1.** ANOVA table of α-diversity analysis. **Table S2.** Taxonomy, OTU ID, and counts of Trifolium RootOTUs with potential genus function and literature references.

Table S3. Temperature and light program used in the climate chamber growth and microcosm experiments. **Table S4.** PCR cycling conditions used in generating amplicons for MiSeq, isolate analysis, and the clone library. (DOCX)

Additional file 2: Sample name, experiment, barcode sequences, and sequence counts of the Trifolium root and soil samples and the simplified community and soil-extract microcosm experiments. (XLSX)

Additional file 3: Command line code and necessary input files needed to replicate bioinformatic analysis. (ZIP)

Additional file 4: R code and necessary input files needed to replicate all statistical analyses and reproduce R-generated figures. (ZIP)

Additional file 5: Unique ID, taxonomy, isolation source, and FASTA sequence of the isolates in the bacteria reference stock. (XLSX)

Abbreviations

OTU, operational taxonomic unit; PCoA, principal coordinates analysis; FAST, Farming Systems and Tillage; FDR, false discovery rate; ANOVA, analysis of variance; HSD, honest significant differences

Declarations

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable

Availability of Data and Materials

The MiSeq 16S sequencing data is stored at the European Nucleotide Archive database (accession no. PRJEB15152). All other files needed to replicate the analysis are available in Additional files 2-5. Raw sequencing files of the bacteria isolates and clone library are available from the authors upon request.

Competing Interests

The authors declare that they have no competing interests

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Authors Contributions

KH, MDVH, and KS conceived of the study, participated in its design, and wrote the manuscript. KH, VRP and KS conducted the experiments and analyzed the data. JCW developed the bioinformatics analysis. All authors read and approved the final manuscript.

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References

1. Curtis TP, Sloan WT, Scannell JW. Estimating prokaryotic diversity and its limits. Proc. Natl. Acad. Sci. U. S. A. 2002;99:10494–9.

2. Hacquard S, Garrido-Oter R, González A, Spaepen S, Ackermann G, Lebeis S, et al. Microbiota and host nutrition across plant and animal kingdoms. Cell Host Microbe. 2015;17:603–16.

3. Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, et al. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature. 2012;488:91–5.

4. Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, et al. Defining the core Arabidopsis thaliana root microbiome. Nature. 2012;488:86–90.

5. Bulgarelli D, Garrido-Oter R, Münch PC, Weiman A, Dröge J, Pan Y, et al. Structure and function of the bacterial root microbiota in wild and domesticated barley. Cell Host Microbe. 2015;17:392–403.

6. Peiffer JA, Spor A, Koren O, Jin Z, Tringe SG, Dangl JL, et al. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. U. S. A. 2013;110:6548–53.

7. Yeoh YK, Paungfoo-Lonhienne C, Dennis PG, Robinson N, Ragan MA, Schmidt S, et al. The core root microbiome of sugarcanes cultivated under varying nitrogen fertiliser application. Environ. Microbiol. 2015;18:1338–51.

8. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, et al. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc. Natl. Acad. Sci. 2015;112:E911–20.

9. Isobe S, Kölliker R, Boller B, Riday H. Red Clover. In: Cai H, Yamada T, Kole C, editors. Genet. Genomics Breed. Forage Crop. Boca Raton, USA: CRC Press; 2014. p. 220–49.

10. Taylor NL, Quesenberry KH. Red Clover Science. Springer Science & Business Media; 1996.

11. Dias PMB, Julier B, Sampoux J-P, Barre P, Dall'Agnol M. Genetic diversity in red clover (Trifolium pratense L.) revealed by morphological and microsatellite (SSR) markers. Euphytica. 2008;160:189–205.

12. Kölliker R, Herrmann D, Boller B, Widmer F. Swiss Mattenklee landraces, a distinct and diverse genetic resource of red clover (Trifolium pratense L.). Theor. Appl. Genet. 2003;107:306–15.

13. De Vega JJ, Ayling S, Hegarty M, Kudrna D, Goicoechea JL, Ergon Å, et al. Red clover (Trifolium pratense L.) draft genome provides a platform for trait improvement. Sci. Rep. 2015;5:17394.

14. Aleklett K, Leff JW, Fierer N, Hart M. Wild plant species growing closely connected in a subalpine meadow host distinct root-associated bacterial communities. PeerJ. 2015;3:e804.

15. Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and plant health. Trends Plant Sci. 2012;17:478–86.

16. van der Heijden MGA, Hartmann M. Networking in the Plant Microbiome. PLOS Biol. 2016;14:e1002378.

17. Schlaeppi K, Bulgarelli D. The Plant Microbiome at Work. Mol. Plant Microbe Interact. 2015;28:212–7.

18. Berg G, Opelt K, Zachow C, Lottmann J, Götz M, Costa R, et al. The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus Verticillium differs depending on plant species and site. FEMS Microbiol. Ecol. 2006;56:250–61.

19. Berg G, Roskot N, Steidle A, Eberl L, Zock A, Smalla K. Plant-Dependent Genotypic and Phenotypic Diversity of Antagonistic Rhizobacteria Isolated from Different Verticillium Host Plants Plant-Dependent Genotypic and Phenotypic Diversity of Antagonistic Rhizobacteria Isolated from Different Verticillium Host Pl. Appl. Environ. Microbiol. 2002;68:3328–38.

20. Zachow C, Tilcher R, Berg G. Sugar beet-associated bacterial and fungal communities show a high indigenous antagonistic potential against plant pathogens. Microb. Ecol. 2008;55:119–29.

21. Bai Y, Müller DB, Srinivas G, Garrido-Oter R, Potthoff E, Rott M, et al. Functional overlap of the Arabidopsis leaf and root microbiota. Nature. 2015;528:364–9.

22. Lebeis SL, Paredes SH, Lundberg DS, Glavina T, Jones CD. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. Science. 2015;349:1678–81.

23. Hugenholtz P, Pace NR. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. Trends Biotechnol. 1996;14:190–7.

24. Chelius MK, Triplett EW. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. 2001;41:252–63.

25. Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 2013;64:807–38.

26. Zarraonaindia I, Owens SM, Weisenhorn P, West K, Hampton-Marcell J, Lax S, et al. The soil microbiome influences grapevine-associated microbiota. MBio. 2015;6:e02527-14.

27. Liu XY, Wang ET, Li Y, Chen WX. Diverse bacteria isolated from root nodules of Trifolium, Crotalaria and Mimosa grown in the subtropical regions of China. Arch. Microbiol. 2007;188:1–14.

28. Marilley L, Aragno M. Phylogenetic diversity of bacterial communities differing in degree of proximity of Lolium perenne and Trifolium repens roots. Appl. Soil Ecol. 1999;13:127–36.

29. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 2013;31:814–21.

30. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013;41.

31. Tabacchioni S, Chiarini L, Bevivino A, Cantale C, Dalmastri C. Bias caused by using different isolation media for assessing the genetic diversity of a natural microbial population. Microb. Ecol. 2000;40:169–76.

32. Bodenhausen N, Horton MW, Bergelson J. Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. PLoS One. 2013;8:e56329.

33. Yarza P, Yilmaz P, Pruesse E, Glockner FO, Ludwig W, Schleifer K-H, et al. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat Rev Micro. 2014;12:635–45.

34. Faircloth BC, Glenn TC. Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. PLoS One. 2012;7:e42543.

35. Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets. Bioinformatics. 2011;27:863–4.

36. Schirmer M, Ijaz UZ, D'Amore R, Hall N, Sloan WT, Quince C. Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. Nucleic Acids Res. 2015;43:e37.

37. Magoč T, Salzberg SL. FLASH: Fast Length Adjustment of Short Reads to Improve Genome Assemblies. Bioinformatics. 2011;21:2957–63.

38. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal. 2011;17:10–2.

39. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods. 2013;10:996–8.

40. Reddy TBK, Thomas AD, Stamatis D, Bertsch J, Isbandi M, Jansson J, et al. The Genomes OnLine Database (GOLD) v.5: a metadata management system based on a four level (meta)genome project classification. Nucleic Acids Res. 2015;43:D1099–106.

41. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–6.

42. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 2010;7:335–6.

43. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2015.

44. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Ser. B. 1995;57:289–300.

45. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. vegan: Community Ecology Package. 2015.

46. Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD, et al. Picante: R tools for integrating phylogenies and ecology. Bioinformatics. 2010;26:1463–4.

47. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One. 2013;8:e61217.

48. Coombs JT, Franco CMM. Isolation and Identification of Actinobacteria from Surface-Sterilized Wheat Roots. Appl. Environ. Microbiol. 2003;69:5603–8.

49. Lane D. 16S/23S rRNA sequencing. In: E S, Goodfellow M, editors. Nucleic acid Tech. Bact. Syst. New York, NY: John Wiley and Sons; 1991. p. 115–75.

50. Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, et al. Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J. Bacteriol. 1996;178:5636–43.

51. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl. Environ. Microbiol. 2006;72:5069–72.

Additional File 1

Deciphering composition and function of the root microbiome of a legume plant

Supplementary Methods

16S rRNA community profiling

DNA extraction: Upon harvest, the replicate root samples from the climate chamber and natural site growth experiments were flash-frozen in liquid Nitrogen and stored at -20ºC until DNA extraction. The root samples were lyophilized and ground to a powder in 2 mL microcentrifuge tubes with one small tungsten bead and a spoonful of glass sand using a Tissue Lyser II (Qiagen, Hilden, Germany; with 2 cycles of 30 seconds at 30 Hz). Genomic DNA was extracted from 500 mg of root (dryweight) and soil (freshweight) subsamples with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. Extracted DNA was quantified using a Quant-iT Picogreen dsDNA Assay Kit (Invitrogen, Eugene, OR USA) on a Varian Cary Eclipse fluorescence spectrometer (Agilent Technologies, Santa Clara, CA USA) and diluted to 10 ng μL^{-1}.

Generation of sequencing amplicons with PCR: Each 20 µL reaction contained 1x 5PRIME Hot MasterMix (5 PRIME, Gaithersburg, MD USA), 0.3% BSA, 200 nM of each primer (799F, 5'-AACMGGATTAGATACCCKG-3', [1]; 1193R, 5'-ACGTCATCCCCACCTTCC-3', [2]) and 30 ng or 10 ng of DNA template for root and soil reactions, respectively. All reactions were performed in an iCycler instrument (BioRad, Hercules, CA, USA) with the cycling conditions given in Table S4. Quadruplicate reactions were pooled, inspected on a 1% agarose gel, and purified using the NucleoSpin PCR purification kit (Machery-Nagel, Düren, Germany). The entire volume of the purified reaction was loaded on a 1% agarose gel, and the ~450 bp amplicon band was cut from the gel. The gel slices were purified using Ultrafree-DA centrifugal filter units (Millipore, Billerica, MA, USA) and quantified using the Picogreen assay described above. The individual samples were pooled in equal amounts into a single 2 mL tube. The volume of the library was reduced with the NuceloSpin PCR purification kit and further purified twice using the Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA).

16S rRNA sequencing: Preparation of the amplicon library for community profiling was conducted as follows: The TruSeq DNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) was used following the manufacturer's instructions. Briefly, the amplicon samples were end-repaired and polyadenylated. TruSeq adapters containing the index for multiplexing were

ligated to the amplicon samples. The ligated samples were run on a 2% agarose gel and the desired fragment length were excised (50bp +/- the target fragment length). DNA from the gel was purified with MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). Fragments containing TruSeq adapters on both ends were selectively enriched with PCR using 4 cycles. The quality and quantity of the enriched libraries were validated using Qubit® (1.0) Fluorometer and the Tapestation (Agilent Technologies, Santa Clara, CA USA). The libraries were normalized to 4nM in Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20. The library was sequenced on the Illumina MiSeq Personal Sequencer (Illumina, San Diego, CA, USA) using a 600 cycle v3 Sequencing kit (Cat n° MS-102-3003), Paired-end 2x 300 bp sequencing mode.

Normalization of OTU counts with rarefaction

Because we found significant differences between the mean sequencing depths per sample group (Kruskal-Wallis test, p=0.008; Figure S10), we chose to rarefy the OTU table and tested for differentially abundant OTUs using non-parametric Mann-Whitney-U tests. This approach is recommended by Weiss *et al.,* [3] who demonstrated that rarefying erases artifacts due to the different sequencing depths of sample groups better than other normalization techniques. For the detection of differentially abundant OTUs however, rarefied data precludes the use of dedicated statistics such as DESeq2 [4] or metagenomeSEQ [5]. However, non-parametric tests on rarefied data offer robust and specific detection of differentially abundant OTUs [3].

Statistical analysis of community profiles

All analyses were performed using R v3.1.2 [6] and the specific R and Biocondutor packages listed below. The OTU and taxonomy tables were filtered to exclude OTUs classified as Eukaryotes, chloroplasts, and mitochondria. The OTU table was rarefied to 20,000 sequences per sample using the R-package *vegan* v 2.3-5 [7]; Figure S10, see method above). We then calculated the relative abundance of each OTU by dividing the number of counts of an OTU in a sample by the total number of counts in that sample, and we expressed the proportions as percentages. All statistical analyses were performed on $\log_2$+1 transformed relative abundance OTU counts.

Alpha and beta diversity: Rarefaction analysis was performed in QIIME v,1.8 [8] on the filtered OTU table (exported from R for this purpose) from 2,000 to 100,000 sequences with a step size of 2,000 and 100 iterations at each sequencing depth. Estimates of alpha diversity (OTU richness and Faith's Phylogenetic Diversity, PD [9] were calculated using the R-package *picante* v1.6-2 [10]. Differences in alpha diversity measures were tested using Two-

way ANOVA with the model *Alpha Diversity Measure ~ Sample Type * Growth Condition.* To quantify the major variance components of beta diversity between root and soil samples, we performed PCoA on weighted UniFrac distances [11] calculated from the phylogenetic tree using PyNAST [12] aligned sequences and FastTree [13] as implemented in QIIME v1.8. The beta diversity analysis was performed with the Bioconductor package *phyloseq* v1.14 [14].

Defining the enriched and abundant members of the root microbiome: We defined the root enriched OTUs (significantly higher abundance in root compared to soil samples) by utilizing non-parametric Mann-Whitney tests, and we considered the OTUs with a >2-fold change between root and soil samples and $P<0.05$ (FDR corrected) to be the root enriched community. We further defined the abundant members of the Trifolium root microbiome (RootOTUs) as OTUs having a mean relative abundance of ≥0.1% across all samples. This abundance threshold is similar to previous studies [15–18]. All *P*-values were adjusted for multiple comparisons with the FDR correction using the Benjamini-Hochberg method [19]. Four RootOTUs could not be taxonomically assigned using SILVA and were assigned using the 16S ribosomal RNA database with NCBI BLAST. The R code and all necessary input files are available in Additional file 4.

Bacteria reference stock

Root processing for bacteria isolation: Freshly harvested 5 cm root fragments of 9 plants were cut into smaller segments of 1-3 cm. The cut and mixed root segments were then divided into three sterile 50 mL tubes containing 25 mL sterile phosphate-buffered saline buffer supplemented with 0.05% Tween 20 (PBS-T buffer) and vortexed at maximum speed for 30 sec. The roots were removed from this first washing step and transferred to new 50 mL tubes containing fresh 25 mL PBS-T buffer and shaken at 28°C and 150 rpm for 20 min. After this second washing step, the root segments were transferred to a sterile Petri dish and divided equally between 24 2 mL microcentrifuge tubes each containing 750 µL sterile PBS-T buffer, one large Tungsten bead (⌀ 2 mm) and one small spoonful of glass beads (⌀ 0.8 mm). The samples were lysed with a TissueLyser II (Qiagen, Hilden, Germany) for two cycles of 2.5 min at 30 Hz. We created the root slurry for plating by pooling the contents of all tubes into one meta-sample and filtering it through a sterile 250 µm sieve to remove the lysing beads and large root debris.

Bacteria isolation: The root slurry was serially diluted and 20 µL of the 10^{-4} to 10^{-7} dilutions were plated onto Flour medium (FM) agar [20] plates amended with 10 µg mL^{-1} Cycloheximide (to inhibit fungal growth; Sigma Aldrich, St. Louis, MO USA). The plates were

incubated at 28˚C for 1-10 days. Single colony forming units were selected and sub-cultured three times on FM plates. The isolates were collected as the bacteria reference stock in 96-well deep-well culture plates containing 1 mL FM medium and were duplicated for PCR-based taxonomy identification (see below) or supplemented with 20 % glycerol (v/v final) for long-term storage at -80°C.

Isolate identification: The isolates were grown in liquid FM in 96-well plates until turbid and a 100 µL subsample taken and centrifuged (5min 16 060 x g) to pellet microbial biomass. The supernatant was removed, replaced with sterile H_2O, and DNA extracted by boiling at 99ºC for 10 min to lyse the cells. The plates were centrifuged (5 min 16,060 x g) to pellet cell debris and the supernatant used as DNA template in PCR reactions. Each 20 µL PCR reaction per isolate contained 1 U Phusion High Fidelity DNA Polymerase, 1x HF buffer, 200µM dNTPs, 300nM of each primer (27F, 5'-AGAGTTTGATCCTGGCTCAG-3', [21]; 1401R, 5'-CGGTGTGTACAAGGCCC-3', [22] and 3 µL of template DNA. All reactions were performed in an iCycler instrument (BioRad, Hercules, CA, USA) with the cycling conditions given in Table S4. PCR amplicons were verified on a 1% agarose gel. The reactions were purified and sequenced using the Sanger method with 1401R as the sequencing primer by Microsynth AG (Balgach, Switzerland).

Quality filtering and taxonomic classification of bacteria isolate sequences: The resulting AB1 sequencing files (available in Supplementary data 5) were converted into FASTQ file format using EMBOSS v6.6.0 [23], and degenerate nucleotides were re-assigned with Seqtk (https://github.com/lh3/seqtk). The sequences were then re-orientated to the 5'-3' direction using FASTX v0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). Sequences were quality filtered by trimming 50 bp from the 5' and 3' ends and then progressively trimming nucleotides from both ends at a mean Phred score <25 (window size 5, step size 2). Finally, sequences < 700bp or with a mean Phred score < 30 were discarded. Quality filtering was performed using PRINSEQ v0.20.4 [24]. Quality sequences were used for taxonomy assignment using the RDP classifier against the SILVA (v119) [25] database as implemented in QIIME v1.8. 23 isolates could not be assigned using SILVA and were further classified against the 16S ribosomal RNA sequences database using NCBI BLAST.

Phylogenetic tree: Bacteria isolate sequences were aligned using the PyNAST algorithm in QIIME v 1.8. The phylogenetic tree file was generated using FastTree imported into R, and visualized using the *plot.phylo* function in the R package *ape* [26].

Mapping isolates to OTUs

To cross-reference cultivation independent and dependent efforts we mapped the 16S rRNA sequences of the isolates in the Trifolium reference stock to the OTU representative sequences obtained from the community profiling. The quality-filtered, full-length sequences of the isolates were trimmed 5‘ of the 799F primer site using FLEXBAR v2.4 [27] and trimmed to 360 bp to identify the same region of the 16S rRNA operon as used for community profiling. The trimmed isolate sequences were then mapped to the OTU representative sequences at ≥97% sequence similarity using UPARSE [28].

Estimating within-nodule diversity with a clone library

Nodule harvest, DNA extraction, clone library preparation: We harvested nodules from a separate Trifolium growth experiment conducted and harvested as previously mentioned, except the harvested roots were preserved in 50% EtOH and stored at room temperature until harvesting of the nodules. We aseptically cut 30 nodules from 10 plants and surfaced sterilized them by soaking for 5 min in 5% household bleach and rinsing thoroughly with sterile distilled H_2O. The 30 nodules were then separated into 3 samples of 10 nodules each. DNA was extracted from each sample with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer’s instructions. An 16S rRNA amplicon for cloning was generated using the PCR primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’; [21] and 1401R (5’-CGGTGTGTACAAGGCCC-3’; [22]). PCR was conducted in 50 µL reactions for each sample and contained 1 U Phusion High Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA), 1x HF buffer, 200 µM dNTPs, 300 nM of each primer and 3 µL of template DNA. All reactions were performed in an iCycler instrument (BioRad, Hercules, CA, USA) with the cycling conditions given in Table S4. PCR amplicons were verified on a 1% agarose gel and the remaining volume purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified 16S amplicons were cloned in vectors and subsequently chemically transformed in One Shot® Mach1™-T1^R Chemically Competent *E. coli* using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Eugene, OR USA) according to the manufacturer’s instructions. Vectors from 96 individually picked colonies were isolated and amplicons sequenced using the Sanger method with the gene-specific primer 1401R by Microsynth AG (Balgach, Switzerland). The raw sequencing files are available from the authors upon request.

Microcosm Experiments

Design and construction of microcosms: We modified Magenta GA-7 boxes (Sigma Aldrich, St. Louis, MO USA) to use as microcosms and filled them with 70 g of a calcined clay The experimental substrate had the following physicochemical characteristics: pH 7.3; 96/0/0 % clay/humus/silt; 0.9/ 4.0/ 44.0 mg/kg N/P/K (measured in 1:10 water extract by Eric Schweizer AG, Thun, Switzerland). The lids of the microcosms were modified to have four holes (⌀ 1.5 cm) to permit air exchange. After autoclaving, they were assembled in a flow bench as follows: three holes were sealed with sterile gas-permeable foil and the fourth one was filled with a microbiological foam stopper (possibility to water the plants with a syringe) (Figure S1a,b).

Soil extract experiment: We prepared a soil extract from the same batch of experimental soil that we used for the other experiments of this study. We aimed at a procedure that extracts the microbiota from a soil, removes structural and nutritional properties of the soil and thereby presents a "clean" microbial inoculum for subsequent experiments. We blended for each a subsample (5 g) of the experimental soil in 10 ml sterile PBS-T buffer in a laboratory blender (Polytron, Kinematica, Lucerne, Switzerland; setting 3 for 30 seconds) and collected the supernatant after centrifugation (2 min 3,220x g). This extraction step was repeated three times and the supernatants combined. The blender was sterilized by soaking in 5% bleach for 5 min, 70% ethanol for 5 min, followed by two rinses in sterile H_2O. We inoculated 200 µL of the soil slurry to 50 mL of 15% Hoagland solution [29] amended with 10 µg mL^{-1} Cycloheximide to reduce fungal growth and incubated for 72 hours in a 28˚C incubator at 150 rpm. We chose this approach to reduce fungal growth, enrich the soil extract for bacteria and because we wanted to expose the soil microbiota to the nutrient conditions of the future microcosm experiments. After this preconditioning step, we centrifuged the liquid cultures (5 min, 3 220x g) to pellet microbial biomass, discarded the supernatant, and then re-suspended the microbiota in 50 mL of fresh 15% Hoagland solution. We estimated the bacterial cell number with plating serial dilutions on FM medium to $1.4*10^9$ cells mL^{-1}. The fresh soil microbiota extract was maintained at 4ºC until inoculation to the microcosm. Aliquots of the soil microbiota extract were sampled for community analysis to quantify the inoculum at the start of the experiment.

In a sterile flow bench, we inoculated 70 mL of 15% Hoagland solution containing $1*10^6$ cells mL^{-1} of soil microbiota to the substrate in the microcosms, planted four Trifolium seedlings in each microcosm, and closed them with the modified lids (Figure S1c). The microcosms were maintained in a climate chamber at constant, light, temperature, and humidity conditions (Table S3), and after 25 days, we collected root (5cm) and substrate samples (Figure

S1d). Finally, we determined the community profiles of soil extract samples (4 independent extractions as described above), root samples, and substrate samples using the approach described above.

Simplified bacteria community experiment: We used four bacteria strains from the reference stock isolate collection to conduct a microcosm experiment to assess their effects on plant growth when inoculated individually or in combination. We performed three replicate experiments, each having 24 microcosms (6 treatments * 4 replicates). In a sterile flow bench, we inoculated the microcosms of each bacteria treatment with 70 mL of 15% Hoagland solution containing $1*10^6$ cells mL^{-1} (OD_{600}). Replicates of the combination treatment were inoculated with 70 mL of 15% Hoagland's solution containing equal amounts of all four bacteria strains to reach a final concentration of $1*10^6$ cells mL^{-1}. 70 mL of nutrient solution without bacteria served as the control. We planted four Trifolium seedlings in each microcosm, and closed them. The microcosms were maintained in a climate chamber at constant, light, temperature, and humidity conditions (Table S3). After 25 days, we collected root (5cm) samples from each replicate microcosm and quantified bacteria colonization on the roots.

Quantification of root bacteria colonization: At the harvest of each simplified community experiment, we determined the level of bacterial colonization in the root. One root from each replicate was cut from the plant and homogenized in 1.5 mL Eppendorf tubes containing 1mL 10mM $MgCl_2$, glass beads (⌀ 0.8mm), and one tungsten bead (⌀ 2mm) with the Tissue Lyser II instrument for 3 minutes at 15Hz. The resulting root slurry was serially diluted from 10^{-1} to 10^{-6} and 10µL of each dilution plated on FM [20] agar plates amended with 10µg mL^{-1} Cycloheximide. The plates were incubated for 24 hours at 28°C before visual identification and counting of individual colonies to determine cell density of the inoculated bacteria.

Statistical analysis of simplified community experiments We assessed the effect of the bacteria treatments in the three replicate experiments using Two-way ANOVA with the following model: *Shoot Fresh Weight ~ Bacteria treatment * Experiment.* Values for shoot fresh weight were log transformed to the assumptions for ANOVA. Significant differences between the different treatments were assessed with Tukey's HSD test and were considered significant at $P < 0.05$. We subsequently determined the proportion of sequences from the tested strains in the experimental inoculum and on the root samples by applying a relative abundance threshold of ≥0.1% across the inoculum and root samples separately.

Supplementary Results

Exploring nodule diversity with a clone library

The high abundance of *OTU1* in the overall root community suggested that these sequences originated from rhizobia bacteria present in root nodules. We tested this hypothesis by Sanger sequencing of a 16S rDNA clone library prepared from separately collected and surface-sterilized nodules, and we mapped the nodule-derived sequences to the reference OTUs of the community sequencing. The 95 nodule-derived sequences comprised 10 unique sequences that clustered into three OTUs (Figure S6). The majority of sequences (#91, 6 unique sequences) indeed clustered to *OTU1*, and 3 sequences (all unique) clustered to *OTU3*, a member of the γ-Proteobacteria. A single unique sequence with a 98% match to *Rhizobium leguminosarum* bv. viciae (NCBI BLAST) could not be clustered to a root or soil OTU in our dataset. Besides confirming that *OTU1* represents the rhizobia bacteria in the nodules, we noted some within-nodule diversity at ≥97% sequence similarity (Figure S6).

Microcosms for plant-microbiota experiments

We developed a microcosm system where we grew Trifolium plants in polycarbonate boxes that were supplemented with a calcined clay-based growth substrate and a mineral nutrient solution (Figure S1a-d). We tested the suitability of these microcosms as an experimental system to investigate plant-microbiota interactions. We inoculated soil extract to the microcosms, planted Trifolium, and measured the community assembly on the roots after 25 days of incubation (see Supplementary methods for details). Of note, the microcosms were maintained in the same climate chamber, under the same conditions, and the soil extract was prepared from the same soil batch that we used for the climate chamber experiments with soil-grown Trifolium. Goals of this experiment were to evaluate soil extract as start inoculum compared to native field soil and to define the Trifolium root microbiome in the microcosms. We collected root, substrate, and soil extract samples and determined the bacterial communities using the same approach as described above. The microcosm experiment yielded 156,850 high-quality, non-chimeric sequences with a median of 10,062 (range 1,251 – 20,466) sequences per sample (Additional file 2). For community comparison we sub-sampled the data to 3,000 sequences per sample, removing one substrate sample (only 1,251 sequences) from the analysis.

Initially, we prepared four replicate soil extracts and evaluated them as start inoculum by comparing its community composition to that of the native soil from which it was prepared. To this end, we quantified bacteria OTU richness and found 596 ± 2 OTUs (mean ± s.e.m) in

the experimental soil samples and 544 ± 12 OTUs in the soil-derived extract samples. OTU richness only differed slightly between native soil and soil extract samples (Welch's t-test; t=4, p=0.02 Figure S7a) revealing that our soil extract procedure recapitulated a large portion of the bacterial richness of the natural soil. While native soil and soil extract communities were qualitatively similar, we noted quantitative differences in the taxonomic composition of soil and soil extract communities (Figure S7b). Thus, soil extract is a reproducible start community with bacteria richness comparable to natural field soil, and these results suggest that it presented a suitable tool to inoculate the microcosms.

Subsequently, we analyzed the bacterial communities in the microcosms that formed the soil extract inoculum. We were interested how the root communities in the microcosms compare to those of natural soil-grown Trifolium and whether a selection of bacteria from the substrate of the microcosm to plant roots occurs. We compared the bacterial communities of microcosms (soil extract, substrate, and root samples) and climate chamber (soil and root samples) experiments using PCoA of weighted UniFrac distances to separate the driving factors explaining community differences. There was a distinct separation along PCo axis 1 (explaining 66.6% of the overall variation) between the microcosm and natural soil-grown root samples (Figure S9a). Because the roots in the microcosms were exposed to basically the same microbiota as the roots grown in native soil, this indicates that the composition of the root communities primarily responds to the different physicochemical properties of the growth environment. PCo axis 2 explained 16% of the overall variation and separated the soil and soil extract samples from the root and the microcosm substrate samples. We interpreted the clustering of soil extract and natural soil samples as further support that the soil extract preparation procedure resulted in a "soil-like" start community for the microcosm experiments. This experiment also provided insights into community dynamics when a soil microbiota is introduced into the microcosms: the soil extract inoculum and the microcosm substrate samples clustered distantly and clearly apart from each other in the ordination space revealing that the introduced soil microbiota underwent a substantial community rearrangement in the new environment. Additionally, we noted a subtle separation between clusters of substrate and root samples of the microcosm experiment, suggesting that, like plants cultivated in natural soil, a selection for a root-specific community also occurs in our microcosm system.

In the microcosms, observed richness of the Trifolium root microbiome was 121 ± 11 OTUs (mean + s.e.m). We identified 34 OTUs whose mean relative abundance across all samples was ≥ 0.1%, and these accounted for 95.9% of rarefied microcosm root sequences (Figure S8). The substantial differences between root communities in microcosms and in

natural soil appear to be at least partly the result of a differential recruitment of rhizobia as evidenced by a lower abundance of *OTU1* in microcosm roots compared to the root communities in natural soil (Figure S9b). The soil extract experiment was also intended to identify which OTUs successfully establish under the conditions in the microcosms, which in turn could serve as a rational to choose strains of the reference stock for inoculations to the microcosms (Figure S8).

In summary, the microcosm experiment indicated that soil extract was qualitatively similar to that of normal field soil and served as a diverse start community for microcosm experiments. The inoculated bacteria community underwent substantial community changes in the microcosms, reflected by the distinct clustering of the different sample types. Natural soil and microcosm root communities were qualitatively and quantitatively dissimilar, highlighting the strong community-deterministic effects of the physiochemical characteristics of the growth substrate.

Supplementary Discussion

Root microbiome assembly in microcosms

We produced the soil microbiota with a custom extraction protocol, which was designed to separate the microbes from the physical and chemical components (e.g. nutrients) of the soil we extracted. First, we evaluated whether the soil extraction protocol yielded a microbial inoculum similar in diversity compared to the native soil from which it was extracted. We found with regard to α-diversity that the soil extract was only slightly different in richness from that of the native field soil (Figure S7a). We interpreted this qualitative similarity that our extraction method provides a rich and diverse start community to inoculate microcosms. We found, however, substantial shifts in the taxonomic composition in the soil extract inoculum compared to the natural soil (Figure S7b). We think that the homogenization of the soil as well as the conditioning step (adapting the soil microbes to the plant nutrient solution and incubation with fungicide to counter-select fungi), represent physical and chemical disturbances that disrupt the equilibrium between members of the native soil microbiota and therefore, influence the relative proportions in the inoculum. We assumed that the inoculation of the soil extract to the microcosms subjects the soil microbiota to an ectopic environment (clay substrate and nutrient solution), and we speculated that the community would find a new equilibrium (different community composition) adapted to the new physicochemical conditions. Indeed, we found a distinct clustering between soil extract and substrate samples (Figure S9a), evidencing that the inoculated soil microbiota underwent a substantial community

rearrangement in response to the new conditions in the ectopic environment. Also because of this observation, we conclude that for a start inoculum the presence/absence of taxa is more important than their relative abundances.

The main interest of the soil extract experiment was to follow the assembly of root microbiome in the microcosms and to compare its composition to the one of roots in native soil. Despite that the roots in microcosms and in natural soil were largely exposed to the same soil microbiota and grown under the same controlled climatic conditions, we found that their bacteria communities clustered distantly and clearly apart (Figure S9a). A first explanation is that the strong compositional differences reflect the response of the microbes to the new physicochemical conditions in the microcosms. This explanation conceptually reminds the recurrent observation that the type of soil in which plants root primarily drives the composition of root bacteria communities [16,17,30–32]. In soil, the biogeography of the microbes is mainly determined by edaphic factors and complex interactions between microorganisms [33,34]. In the microcosms, even with the addition of the nutrient solution, the environmental conditions were limited in carbon and nitrogen compared to the native soil and therefore, these conditions potentially exerted a strong selective pressure on the inoculated community and favored those taxa that could quickly adapt to new conditions. Following this logic, edaphic differences between the native organic soil and the predominately mineral substrate would explain the distant clustering and compositional differences of the root communities in soil and microcosm samples (Figure S9a). A second explanation refers to possible effects arising from the difference in duration between the microcosm and natural soil experiments. With the natural soil plants rooted nearly 5 weeks longer than the microcosm grown plants, it is possible that we harvested the microcosm grown plants while dynamic processes of root microbiome assembly were still occurring. Edwards *et al.,* [30] demonstrated that axenic rice seedlings once transplanted into soil begin to assemble an endophyte community within 24h, and that after nearly 2 weeks, rhizoplane and endosphere communities are similar to communities of the same compartment in plants that have been growing for longer. This finding suggests that the bacteria community in microcosm roots would have reached a reasonably representative stage at the time of harvest. However, we observed that root colonization by rhizobia was lower in microcosms compared to soil grown plants (Figure S9b), and therefore future time-course studies are needed to determine the timing when Trifolium roots reach full nodulation and a stable equilibrium in microbiome composition.

We noted in our ordination analysis that the microcosm substrate samples clustered slightly apart from the microcosm root samples (Figure S9a). One interpretation is that we

observed a root selection effect in the microcosms that is induced by exudates secreted from Trifolium roots. The subtle shifts in community compositions may have resulted from certain taxa that proliferated better in response to root exudates, or a competitive advantage of some microbes to colonize the specialized conditions of the root, or a combination of both [35]. Additionally, we noticed that all microcosm root samples clustered closely with each other indicating that the root microbiome established in a reproducible manner in the microcosm conditions. It seems plausible that the homogenous structural and nutritional conditions in the microcosms result in fewer microbial niches compared to a complex natural soil and consequently, microbiome assembly occurs with less variation and possibly also of lower diversity. Supporting such an interpretation, Tkacz *et al.,* [36] conducted rhizosphere microbiota transfection experiments and found that the rhizosphere bacterial diversity and variation between samples generally decreased when nutrient poor sand was used as growth substrate compared to compost, which is rich in organic nutrients. In summary, our soil extract experiment revealed for an introduced microbiota that it undergoes substantial community rearrangement in microcosms, that it reproducibly assembles to a stable root microbiome and also that root selection probably occurs under these conditions.

Supplementary References

1. Chelius MK, Triplett EW. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. 2001;41:252–63.

2. Bodenhausen N, Horton MW, Bergelson J. Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. PLoS One. 2013;8:e56329.

3. Weiss SJ, Xu Z, Amir A, Peddada S, Bittinger K, Gonzalez A, et al. Effects of library size variance, sparsity, and compositionality on the analysis of micrbiome data. PeerJ Prepr. 2015;

4. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:1–21.

5. Paulson JN, Stine OC, Bravo HC, Pop M. Robust methods for differential abundance analysis in marker gene surveys. Nat. Methods. 2013;10:1200–2.

6. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2015.

7. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. vegan: Community Ecology Package. 2015.

8. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 2010;7:335–6.

9. Faith DP. Conservation evaluation and phylogenetic diversity. Biol. Conserv. 1992;61:1–10.

10. Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD, et al. Picante: R tools for integrating phylogenies and ecology. Bioinformatics. 2010;26:1463–4.

11. Lozupone C, Knight R. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. Appl. Environ. Microbiol. 2005;71:8228–35.

12. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics. 2010;26:266–7.

13. Price MN, Dehal PS, Arkin AP. FastTree: Computing Large Minimum Evolution Trees with Profiles instead of a Distance Matrix. Mol. Biol. Evol. 2009;26:1641–50.

14. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One. 2013;8:e61217.

15. Benson AK, Kelly SA, Legge R, Ma F, Low SJ, Kim J, et al. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc. Natl. Acad. Sci. U.S.A. 2010;107:18933–8.

16. Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, et al. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature. 2012;488:91–5.

17. Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, et al. Defining the core Arabidopsis thaliana root microbiome. Nature. 2012;488:86–90.

18. Bai Y, Müller DB, Srinivas G, Garrido-Oter R, Potthoff E, Rott M, et al. Functional overlap of the Arabidopsis leaf and root microbiota. Nature. 2015;528:364–9.

19. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Ser. B. 1995;57:289–300.

20. Coombs JT, Franco CMM. Isolation and Identification of Actinobacteria from Surface-Sterilized Wheat Roots. Appl. Environ. Microbiol. 2003;69:5603–8.

21. Lane D. 16S/23S rRNA sequencing. In: E S, Goodfellow M, editors. Nucleic acid Tech. Bact. Syst. New York, NY: John Wiley and Sons; 1991. p. 115–75.

22. Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, et al. Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J. Bacteriol. 1996;178:5636–43.

23. Rice P, Longden I, Bleasby A. EMBOSS: The European Molecular Biology Open Software Suite. Trends Genet. 2000;16:276–7.

24. Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets. Bioinformatics. 2011;27:863–4.

25. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–6.

26. Paradis E, Claude J, Strimmer K. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics. 2004;20:289–90.

27. Dodt M, Roehr JT, Ahmed R, Dieterich C. FLEXBAR-Flexible Barcode and Adapter Processing for Next-Generation Sequencing Platforms. Biology (Basel). 2012;1:895–905.

28. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods. 2013;10:996–8.

29. Hoagland DR, Arnon DI. The water-culture method for growing plants without soil. Circ. Calif. Agric. Exp. Stn. 1950;347:1–32.

30. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, et al. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc. Natl. Acad. Sci. U.S.A. 2015;112:E911–20.

31. Yeoh YK, Paungfoo-Lonhienne C, Dennis PG, Robinson N, Ragan MA, Schmidt S, et al. The core root microbiome of sugarcanes cultivated under varying nitrogen fertiliser application. Environ. Microbiol. 2015;18:1338–51.

32. Peiffer JA, Spor A, Koren O, Jin Z, Tringe SG, Dangl JL, et al. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. U.S.A. 2013;110:6548–53.

33. Fierer N, Jackson RB. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. U.S.A. 2006;103:626–31.

34. Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G. Microbial diversity and soil functions. Eur. J. Soil Sci. 2003;54:655–70.

35. Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, Dunfield KE. Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am. J. Bot. 2013;100:1738–50.

36. Tkacz A, Cheema J, Chandra G, Grant A, Poole PS. Stability and succession of the rhizosphere microbiota depends upon plant type and soil composition. ISME J. 2015;9:2349–59.

37. Masson-Boivin C, Giraud E, Perret X, Batut J. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? Trends Microbiol. 2009;17:458–66.

38. Hahm MS, Sumayo M, Hwang YJ, Jeon SA, Park SJ, Lee JY, et al. Biological control and plant growth promoting capacity of rhizobacteria on pepper under greenhouse and field conditions. J. Microbiol. 2012;50:380–5.

39. Innerebner G, Knief C, Vorholt JA. Protection of Arabidopsis thaliana against leaf-pathogenic Pseudomonas syringae by Sphingomonas strains in a controlled model system. Appl. Environ. Microbiol. 2011;77:3202–10.

40. Adhikari T, Joseph C, Yang G, Phillips D, Nelson L. Evaluation of bacteria isolated from rice for plant growth promotion and biological control of seedling disease of rice. Can. J. Microbiol. 2001;47:916–24.

41. Benítez MS, McSpadden Gardener BB. Linking sequence to function in soil bacteria: Sequence-directed isolation of novel bacteria contributing to soilborne plant disease suppression. Appl. Environ. Microbiol. 2009;75:915–24.

42. Berg G, Opelt K, Zachow C, Lottmann J, Götz M, Costa R, et al. The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus Verticillium differs depending on plant species and site. FEMS Microbiol. Ecol. 2006;56:250–61.

43. Enya J, Shinohara H, Yoshida S, Tsukiboshi T, Negishi H, Suyama K, et al. Culturable leaf-associated bacteria on tomato plants and their potential as biological control agents. Microb. Ecol. 2007;53:524–36.

44. Loiret FG, Ortega E, Kleiner D, Ortega-Rodés P, Rodés R, Dong Z. A putative new endophytic nitrogen-fixing bacterium Pantoea sp. from sugarcane. J. Appl. Microbiol. 2004;97:504–11.

45. Oliveira CA, Alves VMC, Marriel IE, Gomes EA, Scotti MR, Carneiro NP, et al. Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. Soil Biol. Biochem. 2009;41:1782–7.

Supplementary Figures

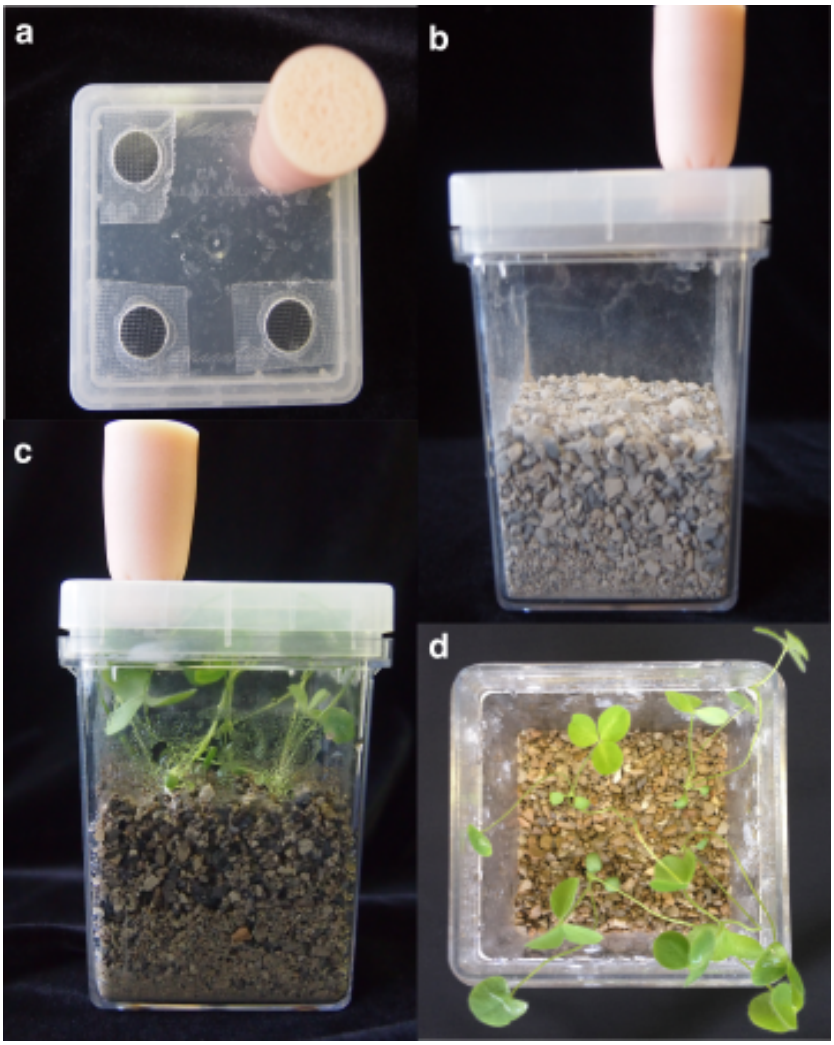

Figure S1: A microcosm system for testing plant-microbe interactions. (a) Three of the four holes in the lid are covered with a sterile, gas-permeable foil to allow air exchange. The fourth hold is plugged with an autoclaved foam stopper to allow watering of the boxes with a syringe and needle during the experiment. **(b)** The microcosms are filled with calcined clay as a growth substrate. **(c)** The microcosms can support 4 plants during the 25-day experimental period. **(d)** Top down view of 25-day old Trifolium prior to harvest.

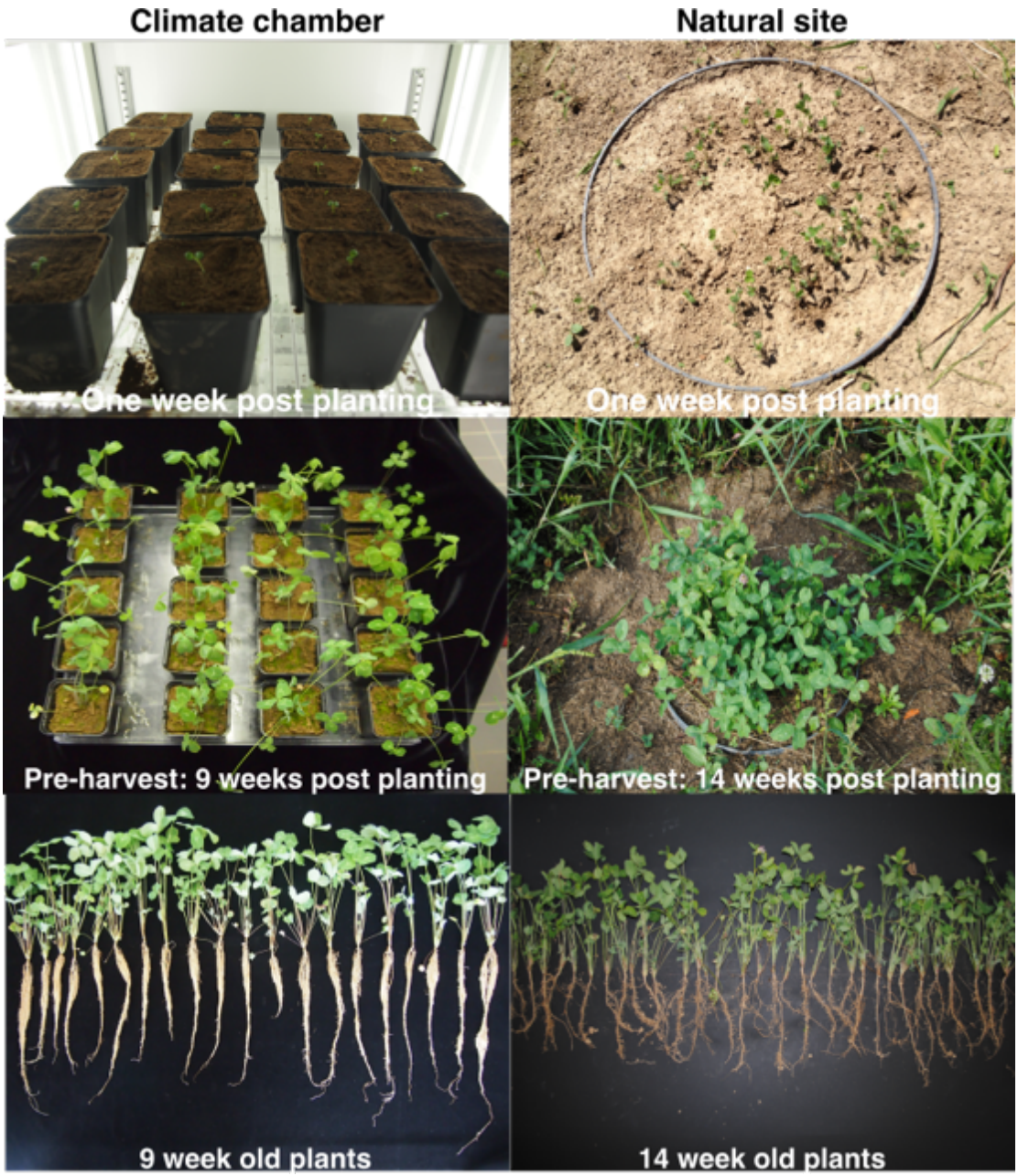

Figure S2: Cultivating Trifolium for root microbiome profiling and reference stock isolation. Trifolium cultivated under controlled conditions in individual pots filled with the experimental soil in the climate chamber after one week (top) and 9 weeks of growth (middle). Trifolium cultivated in the experimental soil under natural conditions after one week (top) and 14 weeks of growth (middle). Plants were harvested when they reached a similar phenotypic stage (bottom photos).

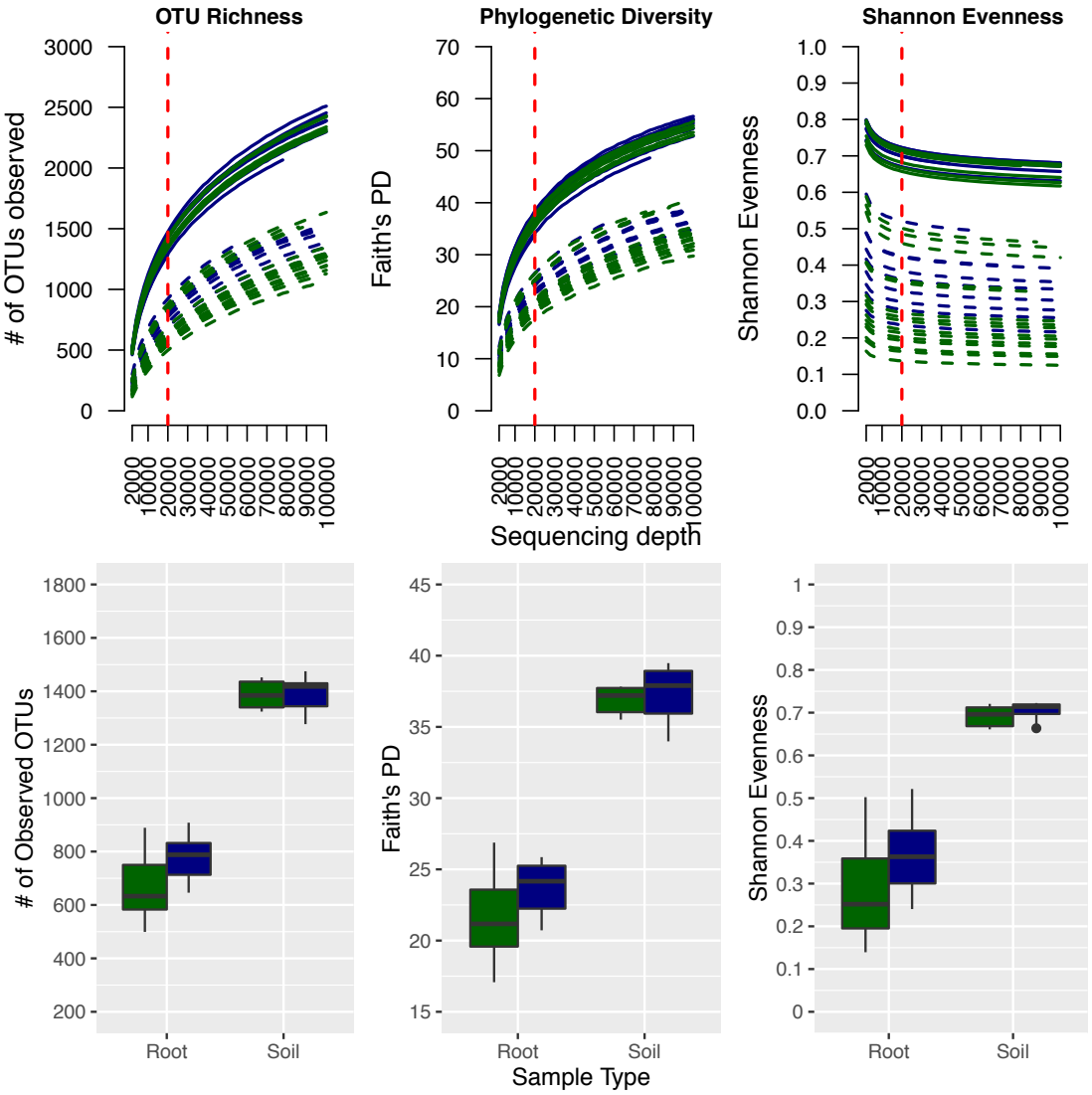

Figure S3: Effects of sample type and growth condition on within sample diversity, as measured by OTU richness, Faith's Phylogenetic Diversity and Shannon Evenness. The rarefaction curves in the first row are means of 100 iterations from 2,000 to 100,000 sequences per soil sample (solid lines) and root samples (dashed lines) in the climate chamber (green) and natural site (blue). The red dashed line indicates the rarefaction depth of 20,000 sequences per sample applied to the dataset. The boxplots in the second row show the alpha diversity measures for the rarefied dataset at 20,000 sequences per sample. The climate chamber (green) and natural site (blue) growth conditions are indicated within each sample type. The results of the Two-way ANOVA of the effects of sample type and growth condition are show in Table S1.

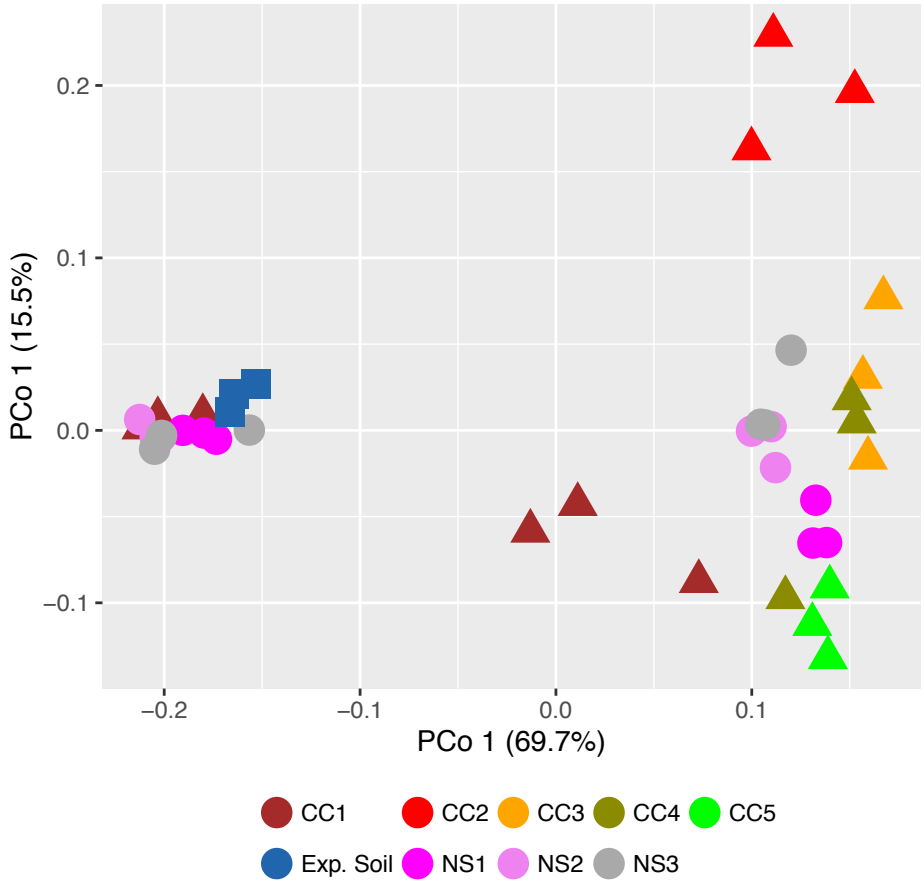

Figure S4: Experimental variation in climate chamber root samples. Same unconstrained PCoA ordination as depicted in Figure 2 in the main text colored by individual growth experiment in the climate chamber (CC1-CC5) or experimental plot in the natural site (NS1-NS3).

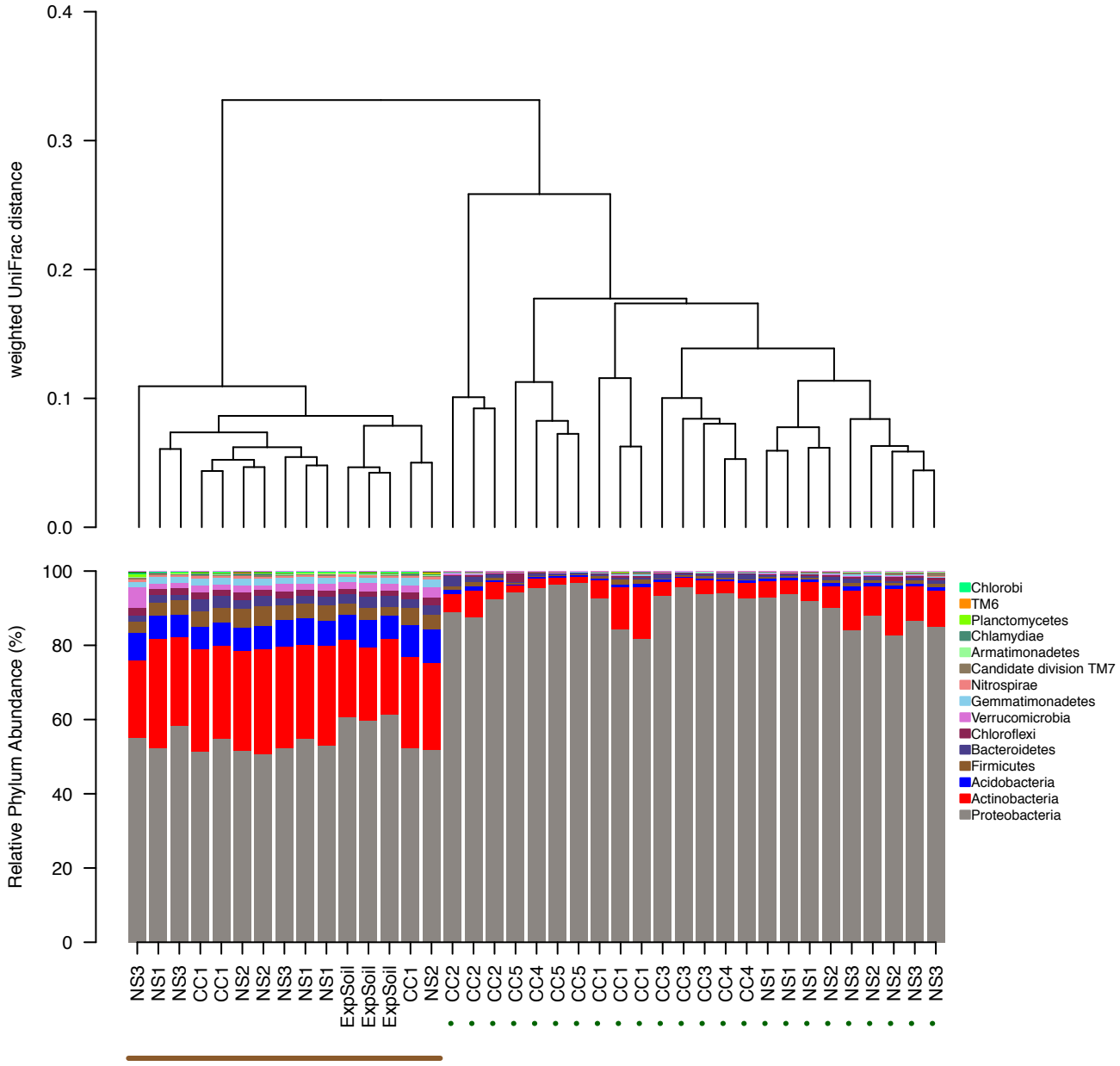

Figure S5: Differences in β-diversity linked to differences in taxonomy. The dendrogram of weighted UniFrac distances demonstrates the distance between root and soil samples. The stacked barplots show the relative abundances of the 15 most abundant phyla in the soil samples (solid brown line) and the root samples (dashed green line) of both natural site (NS) and climate chamber (CC) growing conditions.

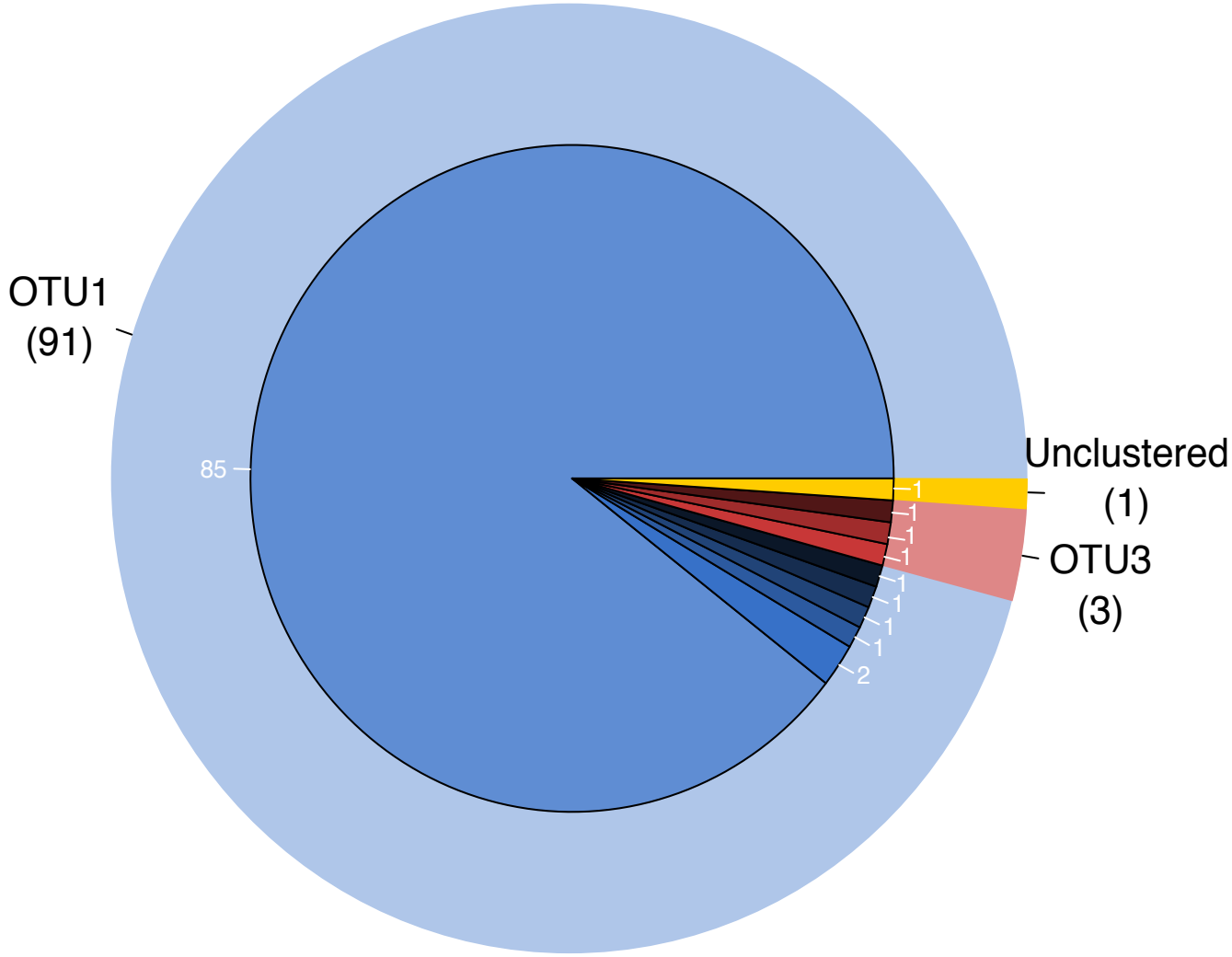

Figure S6: *OTU1* inhabits root nodules of *Trifolium*. The outer ring represents the number of sequences from the clone library clustering to an OTU from the community profile. The inner ring represents the number of unique sequences within the respective OTU.

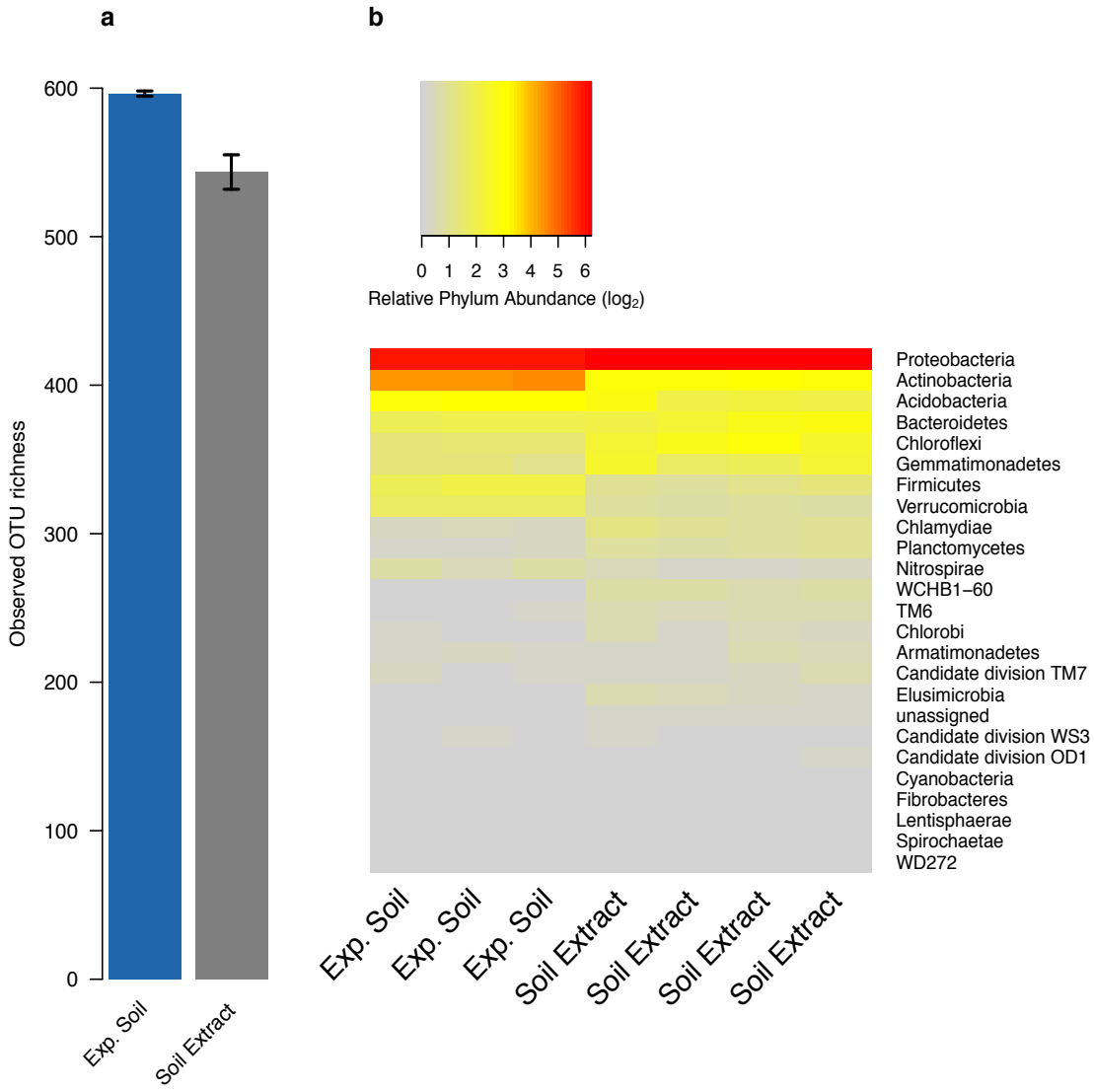

Figure S7: The soil extract captures the diversity of the experimental soil. (a) Means + s.e.m of observed OTU richness of experimental soil samples (n=3) and soil extract inoculum from the microcosm experiments (n=4). **(b)** Heatmap comparing the relative abundances of the shared phyla between the unplanted experimental soil and soil extract samples.

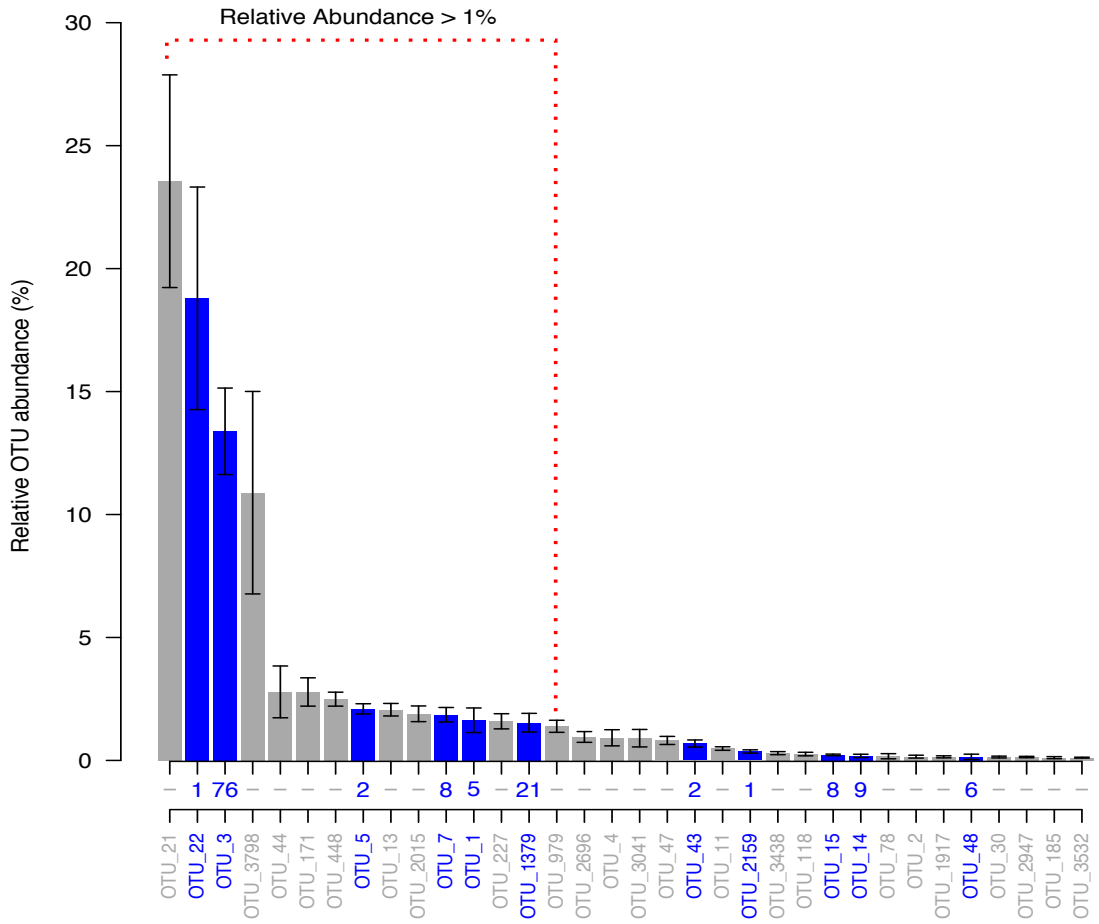

Figure S8: Abundant bacteria community of microcosm roots. Relative abundances of abundant bacteria OTUs (RA > 0.1%) associated with roots of microcosm grown Trifolium inoculated with a diverse bacteria community from the soil extract. Blue bars indicate OTUs for which an isolate is present in the reference stock, with the number of isolates available for each OTU indicated below each bar.

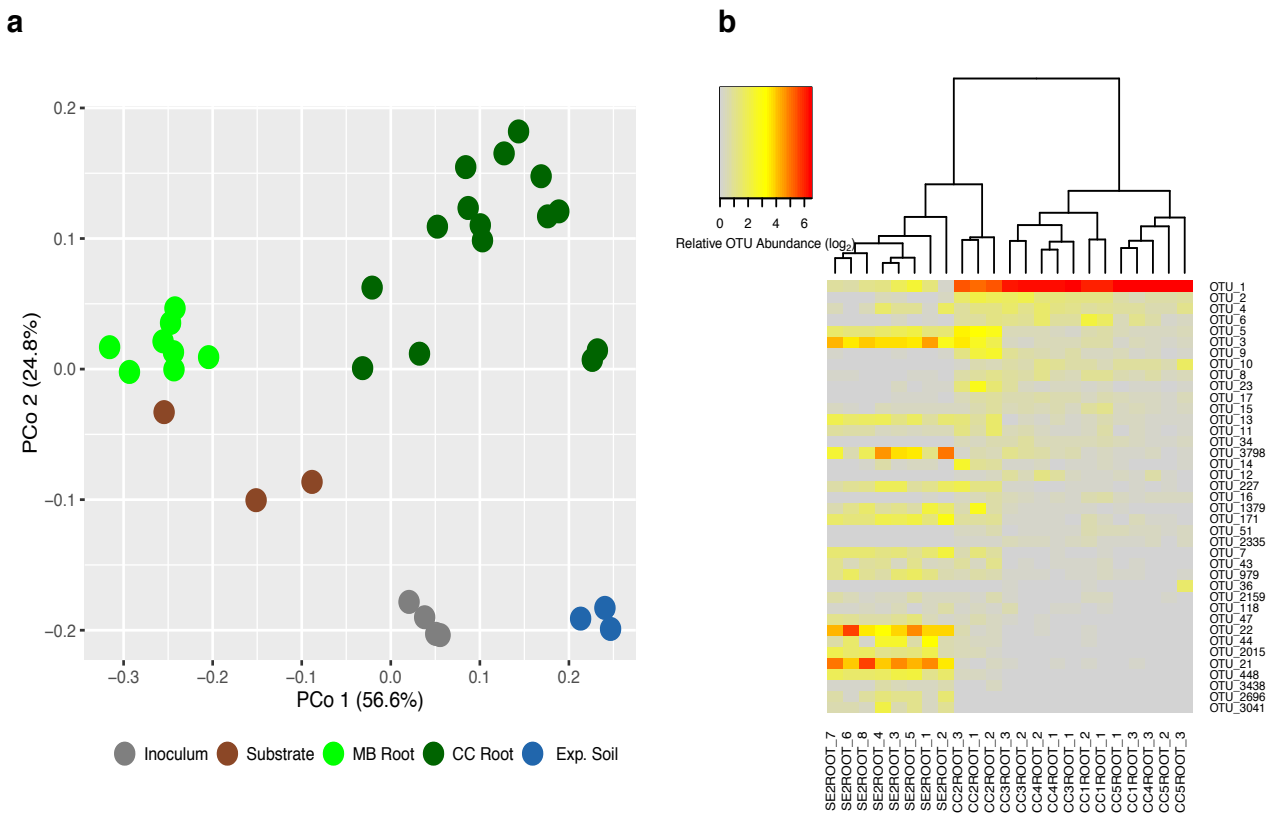

Figure S9: Conditions in the microcosms create unique communities. (a) Unconstrained PCoA plot on weighted UniFrac distances of microcosm inoculum (using soil extracts as inoculum), substrate, and root samples (MB Root), the experimental soil, and the climate chamber root samples (CC Root). **(b)** Relative abundances for OTUs having a mean overall abundance of at least 0.1% across all samples. The soil extract (SEROOT) and climate chamber (CCROOT) sample cluster dendrogram is based upon weighted UniFrac distances. OTUs are ordered according to their relative abundance in climate chamber sample

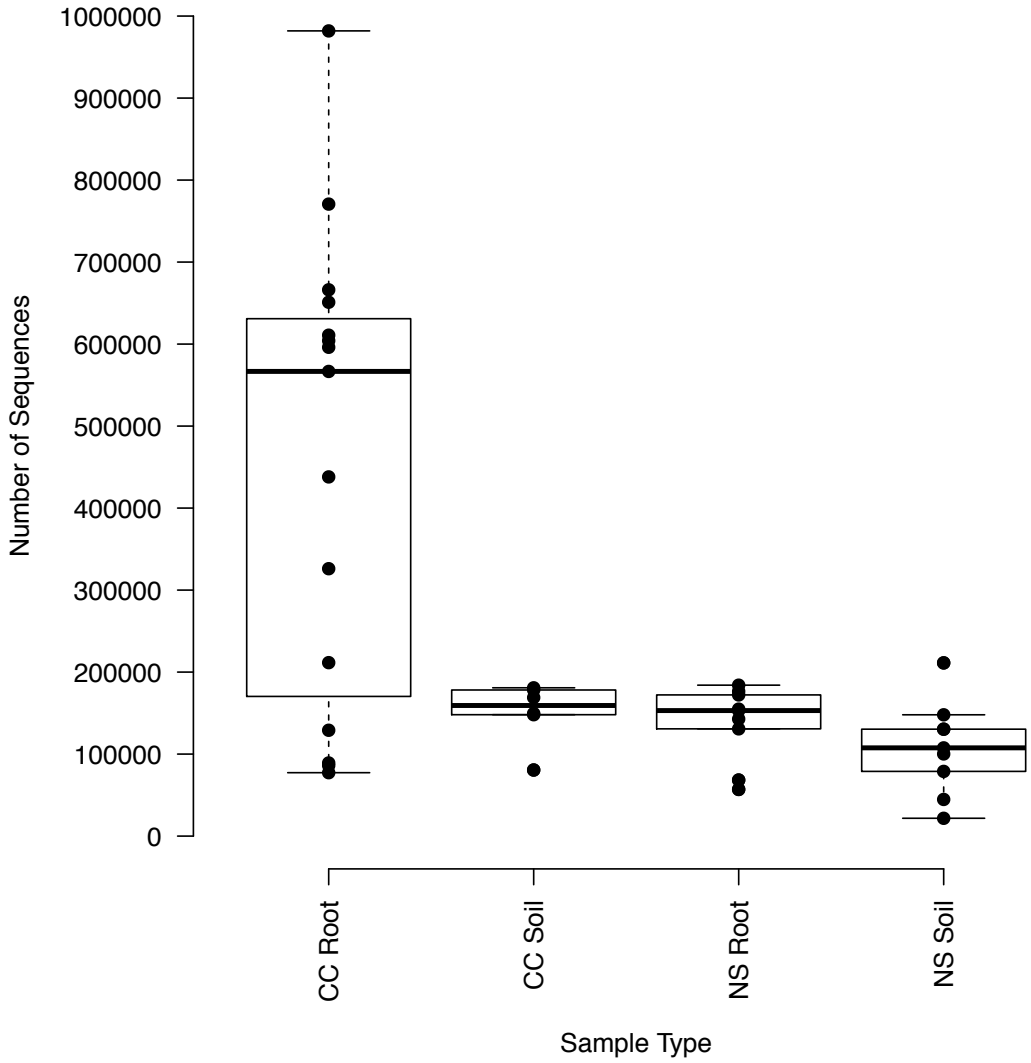

Figure S10: Sequencing depth varied significantly among sample types. Distribution of 16S amplicon sequence counts for climate chamber (CC) and natural site (NS) soil and root samples. Significant differences in sequencing depth required rarefaction of the dataset to 20,000 sequences per sample. See Supplementary Methods for more information.

Supplementary Tables

Table S1: Two-way ANOVA analysis of alpha diversity. ANOVA table showing the effects of sample type (root or soil), growth condition (climate chamber or natural site) and their interaction on alpha diversity in the rarefied community as measured by OTU richness, Faith's Phylogenetic Diversity, and Shannon Evenness. Data are presented in Figure S4.

	OTU Richness			**Faith's Phylogenetic Diversity**			**Shannon Evenness**		
Factor	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***
Sample Type	1, 35	450.43	***<0.001***	1, 35	361.71	***<0.001***	1, 35	175.25	***<0.001***
Growth Condition	1, 35	4.71	***0.04***	1, 35	3.25	0.08	1, 35	3.29	0.08
Sample Type * Growth Condition	1, 35	2.90	0.10	1, 35	1.17	0.29	1, 35	1.35	0.25

Table S2: Taxonomic assignments of the 15 RootOTUs, the abundant (>0.1% RA) and enriched OTUs of the *Trifolium* root microbiome. OTU IDs in bold indicate a culturable member with at least one isolate present in the isolate collection.

Phylum	Class	Order	Family	Genus	OTU ID	Reported benefit to host plant	Reference
Proteobacteria	*α-*	*Caulobacterales*	*Caulobacteraceae*	*Caulobacter*	*OTU44*	-	-
		Rhodospirillales	*Rhodospirillaceae*	*Magnetospirillum*	*OTU37**	-	-
		Rhizobiales	*Rhizobiaceae*	*Rhizobium*	***OTU1***	Nitrogen fixation	[37]
					OTU13		
					OTU10		
					*OTU545**		
					OTU72		
					OTU2335		
		Sphingomonadales	*Sphingomonadaceae*	*Novosphingobium*	*OTU52*	Bacterial disease suppression	[38]
				Sphingomonas	*OTU47*	Bacterial disease suppression	[39]
					*OTU93**	Plant growth promotion	[40]
	β-	*Burkholderiales*	*Comamonadaceae*	*Pelomonas*	*OTU2*	Fungal pathogen antagonism	[41]
	γ-	*Enterobacteriales*	*Enterobacteriaceae*	*Pantoea*		Fungal pathogen antagonism	[42,43]
					OTU286	Nitrogen fixation	[44]
					OTU48	Inorganic P solubilization	[45]
Firmicutes	*Clostridia*	*Clostridiales*	*Syntrophomonadaceae*	*Syntrophomonas*	*OTU28**	-	-
					Total: 15		

***** Taxonomy assignment with NCBI BLAST

Table S3: Growth program for the climate chamber growth experiments. The relative humidity was maintained at 60% during all experiments.

Time	Temp (ºC)	Light Intensity
0600	16	Light 1
0615	16	Light 2
0630	16	Light 3
0645	16	Light 4
0700	25	Light 5
2100	20	Light 4
2115	20	Light 3
2130	20	Light 2
2145	20	Light 1
2200	16	Light 0

Table S4: PCR cycling conditions. Cycling parameters for the generation of the amplicons for MiSeq sequencing for the community profiling, Sanger sequencing for the identification of the reference stock isolates, and the Trifolium root-nodule clone library.

MiSeq Amplicons				**Isolate Identification**				**Clone Library**			
Step	Temperature	Time	Cycles	Step	Temperature	Time	Cycles	Step	Temperature	Time	Cycles
1	98ºC	30sec	1x	1	94ºC	2min	1x	1	98ºC	30sec	1x
2	98ºC	10sec		2	94ºC	30sec		2	98ºC	10sec	
3	54ºC	15sec	30x	**3**	52ºC	30sec	30x	3	52ºC	15sec	30x
4	72ºC	45sec		4	65ºC	30sec		4	72ºC	45sec	
5	72ºC	10min	1x	5	65ºC	10min	1x	5	72ºC	10min	1x
6	15ºC	hold		6	15ºC	hold		6	15ºC	hold	

Chapter 4:

Ecosystem functioning under bacteria and fungi dominated soil communities

unpublished work

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Abstract

Bacteria and fungi comprise the vast majority of microbial life in soil and play important roles in multiple ecosystem functions. Soil disturbances, such as managing soils for agriculture, can induce changes in soil bacteria and fungi community richness and composition, and it is still poorly understood how these changes might affect individual ecosystem functions and overall multifunctionality. Here, we inoculated individual bacteria and fungi isolates from reference stock collections, both separately and in a combined treatment, into microcosms filled with autoclaved soil with the aim of creating bacteria dominated, fungi dominated, and mixed community microbial treatments. We tested effects of these treatments, plus a positive (field soil was used as inoculum) and negative control (sterilized inoculum), on four individual ecosystem functions and overall ecosystem multifunctionality in a microcosm monoculture grassland and characterized the total bacteria and fungi community in each microcosm at the end of the experiment using amplicon sequencing. 69% of inoculated bacteria and 87% of inoculated fungi isolates could be mapped to an OTU in the community profiles based on sequence similarity. However, we also found sequences of the inoculated isolates in microcosms receiving no microbial inoculum, possibly due to an artefact of sterilization resistant DNA captured by the community sequencing or outside contamination. Richness and cumulative relative abundance of OTUs mapping to inoculated fungi were significantly higher in the fungi-only and mixed community treatments, but we detected no such differences in the treatments inoculated with bacteria. Despite no differences in total bacteria or fungi OTU richness between the treatments, with the exception of the positive control, we found addition of different inocula induced shifts in microbial community composition and influenced some of measured ecosystem functions. Litter decomposition was, on average, 40% higher in the positive control treatment, in line with previous findings that higher microbial diversity improves litter decomposition. The composition of the mixed community treatment reduced biomass production by 14% compared to the control treatment and exhibited lower ecosystem multifunctionality when all ecosystem functions were considered together. Overall, our results

demonstrate how isolate collections and model experimental systems can be combined to explore microbial contributions to ecosystem functioning, but also indicate there are still considerable challenges to overcome. We discuss these challenges and ways in which the microcosm system could be improved in future studies.

Keywords: bacteria, fungi, inoculation, microcosm, ecosystem functioning, multifunctionality

Introduction

There is widespread agreement in ecology that biodiversity enhances an ecosystem's resilience to disturbance and its ability to capture and retain necessary resources, produce biomass, decompose, and ultimately recycle biologically essential nutrients [1,2]. The effects of manipulating biodiversity are often investigated on these individual ecosystem functions, or considered on multiple ecosystem functions collectively, so-called ecosystem multifunctionality [2,3]. Many of the conclusions about the relationship between biodiversity and terrestrial ecosystem (multi)functionality have been drawn based upon manipulation of diversity in aboveground communities [4,5]. More recently, however, focus has shifted to exploring the links between belowground soil diversity and its effects on individual ecosystem functions and overall multifunctionality, as understanding of the topic is much less developed [5].

Soils are teeming with communities of bacteria and fungi and can contain millions of individual microbes with hundreds to thousands of different species in a single gram of soil [5]. These soil microbes play an important role in a number of important ecosystem functions, including decomposition, nutrient cycling, plant growth promotion, and protection against pathogens [6]. Consequently, belowground diversity largely determines the productivity of terrestrial ecosystems [7]. Bacteria and fungi normally comprise > 90% of the microbial biomass in soils [8], and most terrestrial ecosystems are dominated by either bacteria or fungi depending on the environmental conditions [9]. However, evidence suggests that soil disturbance, such as management of soils for agriculture, can result in shifts in soil microbial community composition. For example, heavily disturbed soils are thought to be bacteria dominated because physical soil disturbance can negatively affect soil fungi communities through the destruction of hyphal networks [10]. Alternatively, it is thought that less soil disturbance can promote fungal communities and their hyphal networks, potentially improving ecosystem functions like nutrient transport [11]. Thus, a novel question is how ecosystem

functioning and overall multifunctionality will change if soil bacteria and fungi dominance is manipulated.

Previous studies have experimentally investigated effects of altering bacteria or fungi communities on ecosystem functions like plant productivity, nutrient cycling and losses, and decomposition. However, most have focused on manipulating the presence or diversity of specific bacteria or fungi groups like arbuscular mycorrhizal fungi (AMF) [12–15], N-fixing rhizobia bacteria [16], or decomposing fungi [17]. Only recently have studies begun to manipulate several microbial groups together in one experiment, highlighting, for example, the importance of both AMF and rhizobia in legume productivity and nutrient acquisition [18] or general soil biodiversity in the relationship between plant productivity and diversity [19,20]. However, the role of many other microbes in ecosystem functioning remains poorly understood [7]. Therefore, there is still a need for studies that manipulate both bacteria and fungi together in one experiment [21] and test if certain ecosystem functions are correlated with the abundance of specific bacterial or fungal taxa.

A targeted investigation of the function and importance of different bacteria and fungi for various ecosystem functions requires a contained experimental system in which communities of bacteria and fungi can be manipulated without contamination from the outside and a number of ecosystem functions can be simultaneously assessed [7]. Previous studies have altered microbial diversity in microcosm systems using a variety of methods including soil fumigation [22], inoculation of diluted soil suspensions [23,24], and sieving soil through progressively smaller sieves [19,25]. Another suggested approach for functional examination of microbial communities relies on using microbiological techniques to build reference stocks of bacteria and fungi isolates that can be inoculated subsequently into microcosms [26]. Recent works have combined bacteria isolate collections and microcosm systems to investigate bacteria community assembly [27,28]. However, these studies did not include fungi, and the microcosms used were not designed to collect data on multiple ecosystem functions like biomass production, decomposition, or nutrient losses. It has also been demonstrated that only ~50% of bacteria inoculated into microcosms can survive and become abundant [27]. Thus, determining which bacteria and fungi can establish and proliferate under artificial microcosm conditions is important for testing which microbial taxa may contribute to specific ecosystem functions and designing future experiments.

Here, we aimed to manipulate soil bacteria and fungi communities and assess the effects on ecosystem functioning in microcosms planted with a monoculture grassland. To create different communities, we inoculated 48 bacteria and 45 fungi isolates from reference stock

collections, both separately and in a combined treatment, into microcosms filled with sterilized soil. Sterilized, non-inoculated soil and unsterilized field soil served as a negative and positive control, respectively. To maintain the microbial community treatments and reduce outside microbial contamination, we conducted the experiment in specially designed, sealed microcosms. We planted *Lolium multiflorum* and subsequently quantified aboveground biomass production, litter decomposition, leaching volume, and N loss via N_2O emissions. Additionally, we characterized the soil bacteria and fungi community in each microcosm at the end of the experiment using DNA sequencing. We specifically asked: (1) Can bacteria and fungi isolates establish in the microcosms after inoculation into autoclaved soil? (2) Does inoculation of these isolates alter the total richness and composition of bacteria and fungi communities in the microcosms? (3) Do the different microbial community treatments affect the individual ecosystem functions and overall ecosystem functioning in the microcosms?

Materials and Methods

Soil collection and processing: We collected a natural experimental soil from the area outside the experimental plots of the long-term Farming Systems and Tillage (FAST) experiment (47°26'20" N 8°31'40" E; see [29] for a full description). In March 2013 and April 2014, we manually excavated three 1 m^2 plots to a depth of 30 cm. The top layer of vegetation (5 cm) was removed and the remaining bulk soil was collected, passed through a 2 mm sieve, homogenized and stored at 4°C until use. Soil collected in 2013 was used for plant growth experiments to produce root material for bacteria and fungi isolation. Soil collected in 2014 was mixed with sand and used to fill the microcosms in the microcosm experiment.

Processing of Trifolium root samples for bacteria and fungi isolation: The bacteria and fungi isolates used for the microbial inocula were isolated from roots of *Trifolium pratense*, commonly known as red clover, collected from the areas outside the experimental plots at the FAST site. Trifolium plants were also cultivated in a natural growth experiment in the plots from which the experimental soil was collected or cultivated in the experimental soil in multiple climate chamber experiments under controlled climate conditions. A detailed overview of the plant growth experiments used to generate the root samples for isolation, as well as the bacteria isolation protocol, PCR and sequencing methods, and sequence processing steps is presented in Chapter 3 [30].

Root samples for fungi isolation were collected from five separate climate chamber growth experiments and Trifolium individuals sampled from the FAST site. Plants harvested

from the growth experiments were removed from their pots and the roots shaken to remove bulk soil. Naturally collected individuals were excavated from the field with a hand shovel, shaken to remove bulk soil, and placed in a plastic bag. In the lab, all root samples were rinsed with distilled H_2O to remove the loosely adhering soil particles, and 3-5mm root fragments were cut from the lateral roots into a dish of sterile distilled H_2O with a pair of flamed scissors. Under a sterile bench, the root fragments were surface sterilized by agitating in 95% EtOH for 15s, 30% H_2O_2 for 15s, and finally two separate rinses in sterile distilled H_2O.

Fungi reference stocks: Three sterilized Trifolium root fragments per plate were placed on modified Mathur's Medium agar (MMA) [31] or Malt Extract agar (MEA) (Sigma Aldrich, St. Louis, MO USA) plates amended with 15µg/mL oxytetracycline (Sigma Aldrich, St. Louis, MO USA) to inhibit bacteria growth. Additionally, several isolates were isolated from a bulk soil solution created by shaking 5g of the experimental soil with 10mL of sterile phosphate buffered saline (PBS) solution in a 15mL tube. This soil slurry was serially diluted from 10^{-1}-10^{-6} with PBS, and 50µL of the 10^{-3}-10^{-6} dilutions were spread on MMA plates and spread around with a sterilized glass spreader. All plates were incubated at 25ºC until single hyphae were visible on the plate surface. Small fragments of individual hyphae were cut from the plates with a sterilized scalpel and sub-cultured at least three times on MMA or MEA plates. The isolates were subsequently re-plated for PCR-based taxonomy identification (see below) or preserved to create the fungi reference stock. For this, re-plated isolates were allowed to grow until fungal biomass covered the plate. Under a sterile bench, ten plugs of each isolate were punched out from the plate with a flamed cork borer (ø 2.5mm). Five plugs were placed in a 2mL cryogenic tube (Thermo Scientific, Waltham, MA, USA) containing 50% glycerol (v/v final) and stored long-term at -80°C. The other five plugs were placed in a 2mL cryogenic tube (Thermo Scientific, Waltham, MA, USA) containing sterile distilled H_2O and stored at room temperature in the dark.

Fungi isolate identification: A small amount of fungal biomass from each isolate was scraped from the surface of the agar plate and placed in a sterile 1.5mL tube. Fungal DNA was extracted with the REDExtract-N-Amp Plant PCR Kit (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. The extracted DNA was used as a template in PCR reactions. Each 20 µL PCR reaction per isolate contained 10µL REDExtract-N-Amp PCR Ready Mix (Sigma Aldrich, St. Louis, MO, USA), 400nM of each primer ITS5 and ITS4 [32], 4 µL of template DNA, and the remaining volume sterile distilled H_2O. All reactions were performed in an iCycler instrument (BioRad, Hercules, CA, USA) with the cycling conditions given in

Table S1. PCR amplicons were verified on a 1% agarose gel. The reactions were purified and sequenced using the Sanger method with ITS5 as the sequencing primer by Microsynth AG (Balgach, Switzerland).

Quality filtering and taxonomic classification of fungi isolate sequences: The resulting AB1 sequencing files were converted into FASTQ file format using EMBOSS v6.6.0 [33]. Sequences were quality filtered by trimming 50 bp from the 5' and 3' ends and then progressively trimming nucleotides from both ends at a mean Phred score <25 (window size 5, step size 2). Finally, sequences < 400bp or with a mean Phred score < 30 were discarded. Quality filtering was performed using PRINSEQ v0.20.4 [34]. Quality sequences were used for taxonomy assignment using the RDP classifier against the UNITE database v7 [35] as implemented in QIIME v1.8 [36].

Clustering isolate sequences in OTUs for inoculum creation: After quality filtering of the isolate sequences, we identified 200 and 214 high-quality bacteria and fungi sequences, respectively (see Chapter 3 [30] for a description of the bacteria sequence pipeline). To define a sequence-based set of diverse bacteria and fungi for inoculating the microcosms, we clustered de-novo the full length bacteria and fungi sequences separately into operational taxonomic units (OTU) at >97% sequence similarity using the *pick_otus.py* command in QIIME v1.8 implementing the *uclust* algorithm [37]. In total, bacteria sequences clustered into 48 OTUs and fungi sequences clustered into 45 OTUs.

Microcosm design: The experiment was conducted in specially designed microcosm growth chambers (Fig. S1). These microcosms were constructed of a polypropylene growth pot (ø 20cm, depth ~25cm) fitted with a clear, polycarbonate plastic lid (height 60cm). To avoid outside microbial contamination, incoming air was filtered through a 0.2µm pore size Millex FG hydrophobic filter (Millipore, Billerica, MA, USA), and water was filtered through 0.22µm pore size Millex GP hydrophilic filter (Millipore, Billerica, MA, USA).

Filling and sterilization of the microcosms: For filling the microcosms, the experimental soil was mixed 1:1 (v/v) with quartz sand, and the soil moisture content (SMC) and water-holding capacity (WHC) of the sand/soil mixture was determined gravimetrically. 500g of quartz stones was first added to the bottom of each microcosm and covered by a 0.5mm propyltex mesh (Sefar AG, Heiden, Switzerland) to improve drainage for future leachate collection. Each microcosm was then filled with 7.6kg dry weight sand/soil mixture, and four 25cm^2 mesh litterbags were buried in the soil in a square shape in the middle of each tube (Fig. S1). Each

bag contained 1g of dried *Lolium multiflorium* litter, harvested from a previous experiment, which had been shredded by hand with scissors. The microcosms were watered to 100% WHC with distilled H_2O and placed in two autoclave bags to prevent accidental contamination in case a bag was later damaged during the experimental setup. The microcosms were then sterilized twice by autoclaving at 121ºC for 99min each time. The autoclave bags were left open during sterilization and the autoclave temperature probe fully inserted into the soil in the center of the microcosm to ensure the temperature reached 121˚C. After the second autoclaving, the autoclave was opened and the autoclave bags were quickly clamped shut by hand. The microcosms were then quickly moved to a sterile bench where both autoclave bags were firmly tied shut. The microcosms, still sealed in the autoclave bags, were placed in the greenhouse for 9 weeks equalize soil chemistry before inoculation and planting. All necessary tubing and filters were placed inside the microcosm lids, the lids covered with aluminum foil, and sterilized twice by autoclaving at 121ºC for 99min each time.

To judge the effectiveness of the autoclaving procedure to remove native soil microbes, we filled a separate microcosm with the experimental sand/soil mixture and collected a ~5g subsample from the middle of the pot. The microcosm was then autoclaved twice at 121˚C for 99 min and another ~5g soil sample collected. We also collected a ~5g soil sample from a microcosm that had been autoclaved and allowed to sit sealed in the greenhouse for 16 weeks. These samples were shaken in separate tubes with 20mL 10mM $MgCl_2$ and serially diluted from 10^{-1}-10^{-7}. 30µL of the 10^{-2} – 10^{-7} dilutions was plated on both FMA and MMA plates and incubated for one week at 28˚C and 25˚C for the FMA and MMA plates, respectively.

To determine if bacteria DNA could survive the autoclaving process, a separate microcosm was filled with the experimental 50/50 sand/soil mixture and autoclaved twice at 121˚C for 99min. A soil sample was collected from the center of the microcosm before and after autoclaving and soil DNA extracted from a 300mg subsample with the NucleoSpin Soil DNA extraction kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The DNA was used in PCR reactions with the general bacteria primers Eub338 and Eub518 [38] producing an ~250bp amplicon with the cycling conditions in Table S1. DNA extracted from a separately prepared single bacteria colony served as the positive control. We also conducted reactions containing DNA from autoclaved soil and positive control DNA to test for the presence of PCR inhibitors released during autoclaving. All reactions were visualized on a 1% agarose gel.

Seed germination for planting: Seeds of *Lolium multiflorum* Lam. var. Daxus were sterilized in a bottle top vacuum filter (Nalgene, Rochester, NY USA) by incubating for 10min in 500mL of 70% EtOH, followed by 10min in 500mL of 5% NaClO, rinsing twice with 500mL of sterile distilled H_2O, and drying for several hours in a sterile flow bench. The seeds were then pre-germinated for ten days on square Petri dishes contained 0.5x Murashige and Skoog basal medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 1% sucrose. The seed germination was staggered by experimental block so that all seeds were the same age at the time of planting.

Inocula creation: Our study compared five different microbial community treatments: bacteria only (*Bac*), fungi only (*Fun*), bacteria/fungi mix (*Mix*), a positive control of unsterilized experimental field soil (*PCon*), and a negative control of autoclaved experimental field soil (*Con*). To create the *Bac*, *Fun*, and *Mix* treatments, we first cultivated the selected representative isolates for each of the 48 bacteria OTUs and 45 fungi OTUs identified in the microbe reference stocks. For those OTUs that had more than one clustering isolate, one isolate was randomly selected. The 48 bacteria and 45 fungal isolates finally used are listed in Table S2 and S3, respectively. The 48 selected bacteria isolates were inoculated from the frozen stocks into individual wells of a 96-well plate containing liquid Flour medium (FM) [39]. The plate was incubated at 28ºC and shaken at 90rpm for five days to ensure enough growth time for slower growing isolates. The wells were supplemented with fresh, sterile FM medium after three days. Subsequently, 50µL of each liquid culture was pipetted onto a FMA plate, 50µL of sterile distilled H_2O was added to facilitate spreading, and the mixture was spread around the plate with a flamed glass spreader. The plates were incubated at 28ºC for up to 21 days, or until bacteria colonies had covered the entire plate. Faster growing isolates were stored at 4ºC until inoculum creation.

For the fungi, one agar plug for each of the 45 selected isolates was taken from the frozen stock and placed on a MMA plate. The plates were incubated at 25ºC for at least ten days in order to ensure enough harvestable material from slower growing isolates. From these plates, plugs were punched out and placed on new MMA plates and incubated at 25ºC for at least 21 days to allow enough harvestable material from slower growing isolates. Faster growing isolates were placed at 4ºC until inoculum creation. For all bacteria and fungi isolates, five replicate plates per isolate were plated to ensure enough biomass for inoculum creation.

The microbial inoculum added to each microcosm was prepared for each replicate microcosm independently. For this, five agar plugs (ø 5mm) from each OTU representative

isolate were punched out from each bacteria or fungi plate and added to a sterile 50mL tube. Inoculum for the *Bac* treatment consisted of 240 bacteria plugs (48 OTUs * 5 plugs per OTU) and, to ensure equal nutrient additions across all treatments, 225 sterile MMA plugs to correct for nutrients in the fungal medium. Similarly, inoculum for the *Fun* treatment consisted of 225 fungi plugs (45 OTUs * 5 plugs per OTU) and 240 sterile FMA plugs to correct for nutrients in the bacteria medium. The *Mix* treatment consisted of 240 bacteria plugs and 225 fungi plugs, and the *Con* and *PCon* treatments 240 sterile FMA plugs and 225 sterile MMA plugs. Sterile 10mM $MgCl_2$ was added to the tube to cover the plugs (~20mL) and the contents blended with a sterile laboratory blender (Polytron, Kinematica, Lucerne, Switzerland; setting 1 for 10 seconds). This slurry was then added to an empty, sterile 250mL flask and the volume was brought up to 200mL with sterile 10mM $MgCl_2$. The experiment consisted of 40 microcosms each randomly assigned to one of the five microbe treatments (8 replicates per treatment). Due to time required for inoculum creation and microcosm set up and planting, the microcosms were randomly assigned into 4 blocks of ten replicates (two replicates per treatment). The inoculum for each block was prepared the day before inoculation and stored at 4ºC overnight.

Microcosm inoculation and planting: The microcosms were inoculated and planted in a sterile flow bench. To ensure better distribution of the liquid microbial inoculum throughout the soil column, 15 holes were made in the soil surface (depth ~10cm) with a flamed glass rod. Subsequently, 200mL of the appropriate treatment inoculum was poured onto the soil surface. In the *PCon* treatment, 400g (dry weight) of the unsterilized experimental soil used to fill the microcosms was added to each microcosm. All other treatments received 400g (dw) of the experimental field soil that had been autoclaved twice at 121ºC for 99min. This added soil was mixed with the ~ top 5cm of the soil with a sterile spoon to further help distribute the inocula around the pot. Twelve pre-germinated Lolium seedlings of approximately equal size showing no visible signs of contamination were then planted in each microcosm in a pre-defined pattern. The microcosms were closed with the polycarbonate lids inside the sterile bench and the small gap between the pot and the lid sealed with black electrical tape (Fig. S1). Due to time constraints, one block of ten microcosms was inoculated and planted per day. The microcosms were placed in the greenhouse in a randomized block and maintained on constant airflow under natural light conditions, supplemented with high-pressure sodium lights to maintain 300W/m^2 during the 16h, 20-25ºC days. Because the unsterilized field soil contained a natural seed bank, microcosms from the *PCon* treatment were briefly opened under a sterile bench four weeks after planting, and non-Lolium species were removed. The microcosms were watered every

48-72h to maintain ~20% SMC by weight (65% of WHC) and were rotated to new, randomly assigned positions in the greenhouse twice during the course of the 15-week experiment.

N_2O measurements: After 13 weeks for experimental blocks 1 and 2 and 14 weeks for experimental blocks 3 and 4, we assessed the effect of the microbial treatments on the production of the greenhouse gas N_2O. The microcosms were fertilized with 25mL of a nutrient solution (486 mM KNO_3, 37 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 50µM KCl, 25µM H_3BO_3, 2µM $MnSO_4$, 2µM ZnO_4, 0.5µM $CuSO_4$, 0.5µM $NaMoO_4$), corresponding to a fertilizer addition of 60kgN/ha and 10kgP/ha. The microcosms were brought up to 100% of WHC with distilled water to provide ideal conditions for N_2O production. The amount of N_2O produced was measured by cycling the air inside each microcosm through a TEI46c-automated N_2O analyzer (Thermo Fisher Scientific, Waltham, MA, USA) for 15 minutes. Each microcosm was sampled immediately after fertilization and subsequently every 12 hours for 96 hours for a total of nine measurements per microcosm. Greenhouse lights were left on during the entire sampling period in an effort to reduce diurnal effects on gas production. This caused the microcosms to dry out faster than usual, and thus the SMC of each tube was again adjusted to 100% WHC before the fifth measurement at 48h post fertilization.

Leaching samples: Leachate measurements were collected after 15 weeks in order to determine the effect of the microbial treatments on N and P leaching. 24 hours before leaching was induced, each microcosm was fertilized with 25mL of a nutrient solution (243mM KNO_3, 37mM KH_2PO_4, 1mM $MgSO_4$, 2mM $CaCl_2$, 50µM KCl, 25µM H_3BO_3, 2µM $MnSO_4$, 2µM ZnO_4, 0.5µM $CuSO_4$, 0.5µM $NaMoO_4$), corresponding to a nutrient addition of 30kgN/ha and 10kgP/ha, and watered to 20% SMC (65% WHC). After 24 hours, each microcosm was watered to 105% WHC, and the valve at the bottom of each microcosm was immediately opened and the leachate allowed to drain into a 2L bottle for 2h. The total amount of leachate was weighed and two subsamples (each ~100mL) were collected for nutrient analysis. One set of samples was stored at 4ºC, and the other was immediately frozen at -20ºC. Due to the amount of time required to perform the leachate nutrient analysis, only leaching volume data are presented in this chapter.

Harvest: The microcosms were harvested after 15 weeks. For this, they were opened inside a sterile bench and the above ground grass biomass was cut by hand at the soil surface. The biomass was dried in paper bags in a drying oven at 60ºC for 48h and weighed. The litterbags were removed, rinsed in distilled H_2O to remove soil particles, dried in paper envelopes at 60ºC for 48h, and weighed. Plant roots were removed from the soil and rinsed gently in distilled

H_2O. The rinsed roots were placed in a sterile Petri dish, and we sampled a 5 cm fragment of the root system corresponding to the soil depth between -1 and -6 cm using a sterile scalpel. These fragments were placed in a sterile 50mL tube and immediately frozen in liquid nitrogen. Bulk soil samples were collected by mixing any remaining soil in the microcosms with a sterilized spatula and removing a ~1kg subsample to an aluminum tray. This subsample was gently mixed again and any large root or plant pieces were removed by hand. A sample of this soil was placed in a 50mL tube and immediately frozen in liquid N.

Soil DNA extraction for community profiling: To characterize the soil bacteria and fungi communities in each microcosm at the conclusion of the experiment, we conducted 16S and ITS amplicon sequencing on soil DNA samples. DNA was extracted from subsamples of the soil collected during the experimental harvest with the NucleoSpin Soil DNA extraction kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Extracted DNA was quantified using a Quant-iT Picogreen dsDNA Assay Kit (Invitrogen, Eugene, OR USA) on a Varian Cary Eclipse fluorescence spectrometer (Agilent Technologies, Santa Clara, CA USA) and diluted to 1ng/μL for use as the template in PCR reactions.

16S and ITS PCR and library preparation: The 16S amplicon library was generated using the PCR primers 799F [40] and 1193R [41] yielding a ~450 bp amplicon spanning the V5-V7 region of the 16S rRNA gene. The primers were adapted with an error-tolerant 6-mer barcode selected from a list in Faircloth and Glenn [42] to allow for multiplexed library sequencing. PCR reactions were performed on a iCycler instrument (BioRad, Hercules, CA, USA) using the 5PRIME Hot Master Mix PCR system (5 PRIME, Gaithersburg, MD USA) with the cycling conditions in Table S1. Each 20 μL reaction contained: 8 μL 5PRIME Hot Master Mix, 0.3 % BSA, 200 nM each primer, 2 ng template DNA, and the remaining volume sterile, distilled H_2O. PCR reactions were conducted in triplicate and pooled together before inspecting 3 μL of each sample on an agarose gel for correct size and absence of contamination in non-template reactions. PCR reactions were then purified using the NucleoSpin Gel and PCR Clean up Kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions, quantified using a Picogreen assay, and pooled together into the sequencing library in equal amounts (100ng/sample).

The ITS amplicon library was generated using the PCR primers ITS1F [43] and ITS2 [32] yielding a ~300bp amplicon spanning the ITS1 region. The primers were adapted with an error-tolerant 6mer barcode selected from a list in Faircloth and Glenn [42] to allow for

multiplexed library sequencing. PCR reactions were performed on an iCycler instrument (BioRad, Hercules, CA, USA) using the 5PRIME Hot Master Mix PCR system (5 PRIME, Gaithersburg, MD USA) with the cycling conditions in Table S1. Each 20 µL reaction contained: 8 µL 5PRIME Hot Master Mix, 0.3 % BSA, 200 nM each primer, 1 ng template DNA, and the remaining volume sterile, distilled H_2O. PCR reactions were conducted in triplicates and pooled together before validation by gel electrophoresis. The reactions were then purified using the NucleoSpin Gel and PCR Clean up Kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions, quantified using a Picogreen assay, and pooled together into the sequencing library in equal amounts (70ng/sample).

Library sequencing: Preparation of the 16S and ITS amplicon libraries for community profiling was conducted as follows: The NebNext Ultra kit (Illumina, San Diego, CA, USA) was used following the manufacturer's instructions. Briefly, the amplicon samples were end-repaired and polyadenylated. TruSeq adapters containing the index for multiplexing were ligated to the amplicon samples. The ligated samples were run on a 2% agarose gel and the desired fragment length was excised (50 bp +/- the target fragment length). DNA from the gel was purified with MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). Fragments containing TruSeq adapters on both ends were selectively enriched with PCR using 4 cycles. The quality and quantity of the enriched libraries were validated using Qubit and Tapestation (Agilent Technologies, Santa Clara, CA USA). The libraries were normalized to 4 nM in Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20. The library was sequenced on the Illumina MiSeq Personal Sequencer (Illumina, San Diego, USA) using a 600 cycle v3 Sequencing kit (Cat n° MS-102-3003), paired-end 2x 300 bp sequencing mode at the Functional Genomics Center Zurich (www.fgcz.ch).

Sequence processing: The raw 16S and ITS MiSeq reads were processed using a custom-developed bioinformatics pipeline. An overview of the processing steps for both datasets is presented in Chapter 2.

Mapping inoculated strains to soil microbial communities: To identify the presence and relative abundance of the inoculated bacteria and fungi isolates that established in the microcosms, we mapped the 16S rRNA and ITS sequences of the inoculated bacteria and fungi strains to the to the representative OTU sequences from the 16S and ITS soil community profiling. The sequences of the 48 inoculated bacteria strains were trimmed at 799F and 1193R to bp with FLEXBAR [44] to identify the same region of the 16S rRNA operon as used for soil community profiling. These trimmed sequences were then mapped to the representative OTU

sequences from the 16S community profiling using UPARSE at ≥97% sequence similarity. The 45 fungi strain sequences were trimmed at the priming site of the ITS2 primer and 20bp were trimmed off the 5' end of the sequence to improve sequence quality before mapping the sequences to the reference OTU sequences from the ITS community profiling with UPARSE at ≥ 97% sequence similarity.

Statistical analyses: All statistical analyses were conducted with R v3.3.0 [45]. The 16S OTU and taxonomy tables were filtered to exclude OTUs classified as Cyanobacteria and mitochondria. Similarly, OTUs whose kingdom was unassigned were removed from the ITS OTU and taxonomy tables. We found significant differences between the mean sequencing depths of the different microbial treatments in the fungi community (Fig. S2). Therefore, we followed the advice of Weiss *et al.,* [46] and rarefied the fungal OTU table to 35,000 sequences per sample, which was the sequencing depth of lowest sequenced sample. We found no such differences in sequencing depth in the bacteria community (Fig. S2), but for consistency across both kingdoms' datasets, we rarefied the bacteria OTU table to 3,900 sequences per sample. We then calculated the relative abundance (RA) of each OTU by dividing the number of counts of an OTU in a sample by the total number of counts in that sample and expressed the proportions as percentages. Rarefaction analysis was performed using the R package *vegan* [47]. Rarefaction curves were constructed by rarefying the bacteria and fungi OTU tables from 0 to 8,000 (bacteria) and 0 to 115,000 (fungi) sequences per sample in steps of 100 sequences and plotting the observed OTU richness at each rarefaction level.

To calculate the observed richness of inoculated OTUs in each microcosm we subset the rarefied bacteria and fungi OTU tables for those OTUs with a mapping inoculated bacteria or fungi isolate (see above). Total observed bacteria and fungi OTU richness was calculated based on all bacteria and fungi OTUs present in the respective rarefied communities. We then tested for differences in inoculated and total OTU richness between the different microbial treatments using linear mixed effects models in the R package *nlme* with the experimental block as a random factor. Pairwise comparisons were conducted with Tukey's Honest Significant Differences test as implemented in the R package *glht* and were considered significant at $p<0.05$.

To determine which of the inoculated bacteria and fungi isolates were able to successfully establish in the microcosms, we tested for differences in the RA of the inoculated bacteria and fungi isolate OTUs between the five different microbial treatments using pairwise Wilcox tests on $\log_2+1$ transformed data. P values were corrected for multiple testing with the

false discovery rate (FDR). In the bacteria community, established OTUs were those that had a significantly ($p<0.05$, FDR corrected) higher RA in the *Bac* and/or *Mix* treatments compared to the *Con* and *Fun*. Similarly, established isolates in the fungi community were those with a significantly higher RA in the *Fun* and/or *Mix* treatments compared to the *Con* and *Bac.*

We performed an unconstrained principle coordinates analysis (PCoA) on Bray-Curtis dissimilarities to quantify the major variance components in the bacteria and fungi communities and subsequently tested for microbial treatment effects using (partial) constrained analysis of principle coordinates (CAP). All ordination analyses were performed using the R package *phyloseq* [48]. Statistical significance of the CAP was assessed using the *permutest* function in the *vegan* package with 10^4 permutations. We tested for differences between the microbial treatment communities with permutational analysis of variance (PERMANOVA) and permutational analysis of multivariate dispersions (BETADISP) using the functions *adonis* and *betadisp*, respectively, in the *vegan* package with 10^4 permutations. Where applicable, pairwise differences between the microbial treatments were assessed with the function *pairwise.perm.manova* from the package *RVAideMemoire* [49].

Effects of the five microbial treatments on the measured ecosystem functions were assessed with linear mixed effect models with the experimental block as a random factor. Treatment effects on plant biomass production were assessed with plant mortality as a co-variate in the model. For decomposition, data from the four litter bags in each microcosm were summed, and decomposition was calculated as the percentage of original litter mass lost. Leaching values were expressed as the percentage of added water lost. Decomposition and leaching data were arcsin transformed to meet assumptions for ANOVA. However, for simplicity, raw data values of all ecosystem functions are plotted. N_2O flux rates were calculated from the increase in N_2O concentration inside the microcosm headspace for each sampling period using:

$$f_x = \frac{\Delta x}{\Delta y} * \frac{pV}{RT} * \frac{1}{m}$$

Where f_x is the flux rate of N_2O, Δx is the measured change in N_2O concentration in [ppm], Δt is the change in time in [min], p is the absolute pressure of gas in [Pa], V the volume of the microcosm headspace in [m^3], R is the universal gas constant, T the ambient greenhouse temperate in [K], and m the amount of dry-weight soil in the microcosm in [kg]. The calculated N_2O flux rates at each sampling time were used to calculate the total amount of N-N_2O lost from each microcosm over the entire sampling period through linear interpolation using the *auc* command from the R package *flux* [50].

We standardized all ecosystem function data by z transformation (overall mean of 0 and standard deviation of 1) and used these values to calculate an ecosystem multifunctionality index for each microcosm. For this, we took the mean of all z-transformed ecosystem function values [51]. Data for leaching and N-N_2O loss were multiplied by -1 because we considered higher values of these functions to reflect a more undesirable ecosystem state. Thus, increasingly negative multifunctionality values reflect a decline in overall ecosystem functioning [19].

Results

Sequencing of bacteria and fungi communities in the microcosms

To determine whether we had successfully manipulated the bacteria and fungi richness and community composition within the experimental microbial treatments, we performed bacteria and fungi community profiling on soil samples collected from each microcosm at the end of the experiment. The bacteria community profiling yielded a total of 241,821 high-quality sequences. Sequence counts ranged from 3,935 to 7,889 sequences per sample, with a median of 6,090. We identified 2,215 bacteria OTUs across all samples. Fungal community profiling of the same soil samples yielded 3,020,511 high-quality sequences, ranging between 35,657 and 114,798 sequences per sample (median: 74,248), and we identified a total of 774 fungal OTUs. We rarefied the bacteria and fungi communities to 3,900 and 35,000 sequences/sample, respectively, which was sufficient to capture most of the observed total OTU richness in each community (see Materials and Methods; Fig. S2).

Recovery of inoculated strain sequences in the microcosms

We determined the recovery rate of inoculated bacteria and fungi strain sequences by clustering the 48 bacteria and 45 fungi isolate strain sequences to the representative sequences from the soil community profiles at ≥97% sequence similarity (see Materials and Methods). In the bacteria community, 33 inoculated isolates (68.8%) clustered to 25 OTUs present in the rarefied bacteria community (see discussion for explanation), while for 15 isolates we did not find a matching OTU (Table S2). In the fungi community, 39 inoculated isolates (86.7%) clustered to 37 OTUs in the rarefied fungi community profile, and for 6 isolates we did not find a matching OTU (Table S3).

Inoculated OTU richness and abundance

We assessed the inoculation success of the bacteria and fungi isolates in each microcosm by comparing the richness and RA of inoculated OTUs across the different

microbial treatments. For this analysis, the data were subset to only the OTUs matching an inoculated bacteria or fungi isolate. In the bacteria community, we found the richness of inoculated OTUs was highest in the *PCon* treatment and significantly higher than that of the *Con*, *Fun*, and *Mix* treatments. However, we noted no significant differences in inoculated OTU richness between the *Bac* and other microbial treatments (Fig. 1a, Table S4). We found the highest proportion of sequences belonging to inoculated bacteria OTUs in the *Fun* treatment, although this was not significantly different from that of the *Bac* or *Mix* treatments. (Fig. 1b, Table S4). Although inoculated bacteria OTU richness was highest in the *PCon* treatment, the total RA of these OTUs was significantly lower than in the *Bac*, *Fun* and *Mix* treatments (Fig. 1b, Table S4).

To determine which of the inoculated bacteria isolate OTUs were able to establish inside the microcosms, we investigated RAs of the 25 inoculated OTUs present in the bacteria community across the five microbial treatments (Fig. 1c, Fig. S4). We noted that some OTUs had a high RA in every treatment, regardless of whether or not they received the bacteria inoculum. Notably, the high RA of inoculated OTUs in the *Con* and *Fun* treatments (Fig. 1b), appeared to largely be the result of two OTUs (bOTU1 and bOTU2; Fig. 1c). In total, these two OTUs comprised 32.4% and 20.5% of rarefied sequences in the *Fun* and *Con* treatments, respectively (Table S2). Pairwise statistical testing of all OTU RAs between the five microbial treatments revealed that there were no inoculated bacteria OTUs with a significantly higher RA exclusively in the *Bac* and/or *Mix* treatments (Fig. S4). Mean RA values for all inoculated bacteria OTU in each treatment and results from the pairwise statistical tests are presented for closer inspection in Table S2 and Fig. S4, respectively.

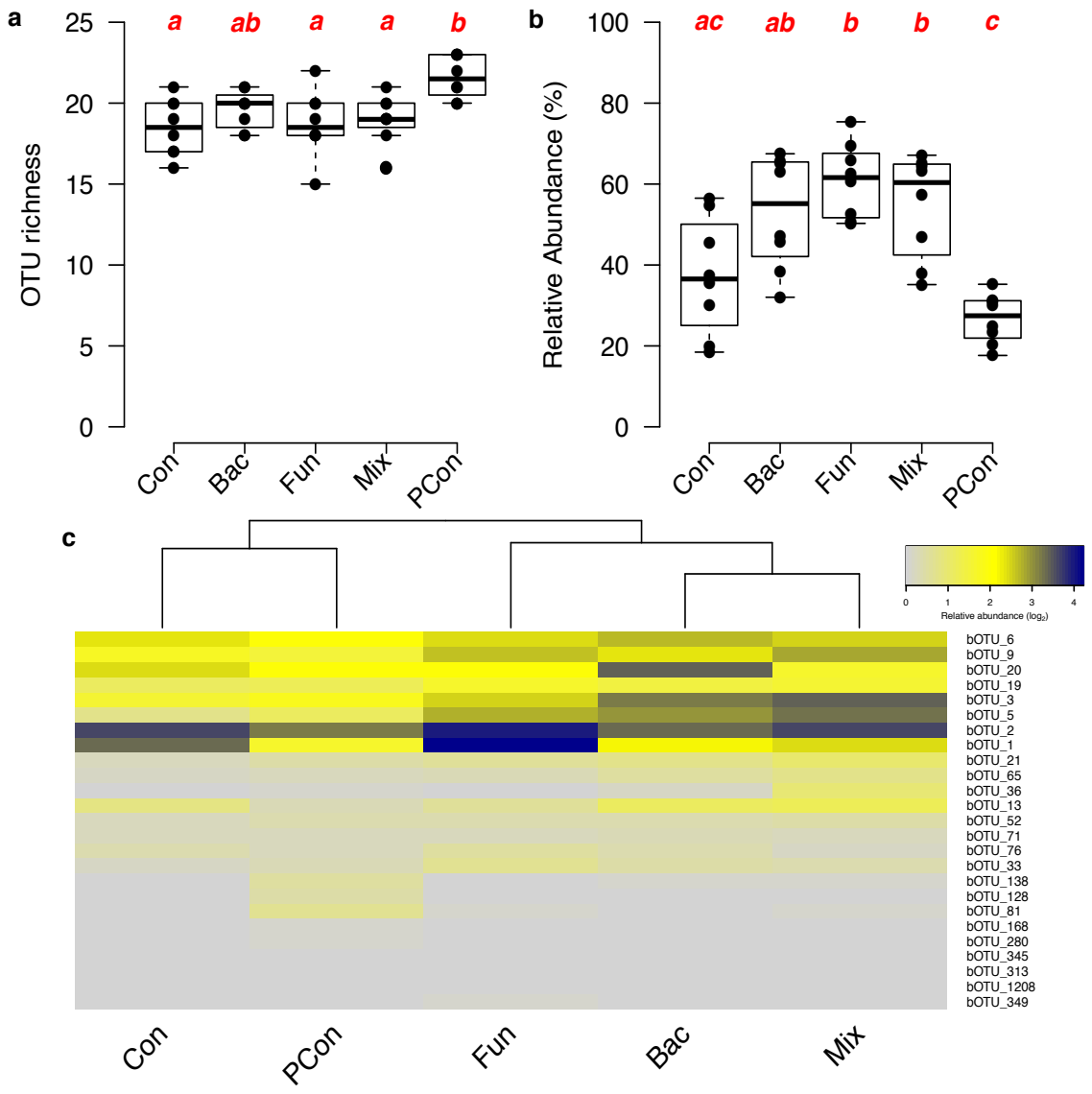

Figure 1: Establishment of bacteria isolate-OTUs in the microcosms**. (a)** Number of inoculated bacteria OTUs present in each microbial treatment. **(b)** Cumulative relative abundance of inoculated OTU sequences in each microbial treatment expressed as percentage of total rarefied sequences. Different letters in **(a)** and **(b)** indicate significant differences between the different treatments as determined by ANOVA and a pairwise Tukey HSD test ($p<0.05$, Table S4)**. (c)** Mean RA of the 25 inoculated OTUs present in the bacteria dataset. Dendrogram reflects a clustering of the inoculated OTU community by Bray-Curtis dissimilarities with method "average." Raw data values and statistical testing for each OTU are present in Fig. S4.

Performing the same analysis on the fungi community revealed that inoculated fungi OTU richness was greatest in the treatments receiving the fungal inoculum (*Mix* and *Fun*), which were significantly higher than in the *Con* and *Bac* treatments (Fig. 2a, Table S4). Differences in the inoculated fungi community were more apparent when looking at the cumulative RA of inoculated fungi isolates across the different treatments (Fig. 2b). Mean RA of inoculated fungi OTUs were generally very low across the non-inoculated treatments, although we noted one outlier in the *Con* treatment. RAs of inoculated OTUs were significantly

higher in the *Fun* and *Mix* treatments, accounting for a mean of approximately 50% and 40% of rarefied sequences, respectively (Fig. 2b, Table S4). Unlike in the bacteria community, pairwise testing of the RAs of the individual inoculated fungi OTUs suggested a number of fungi isolates were able to establish and proliferate in the *Fun* and *Mix* treatments. This was supported by higher mean RAs for a number of inoculated fungi OTUs in these treatments compared to the non-inoculated treatments (Fig. 2c) Indeed, pairwise comparisons of inoculated fungi OTU RAs between the different microbial treatments found 22 inoculated fungi OTUs with significantly higher RAs in the *Fun* and/or *Mix* treatments compared to the non-inoculated *Con* and *Bac* (Fig. S5, Table S3). Mean RA values for all inoculated fungi OTU in each treatment and results from the pairwise statistical tests are presented for closer inspection in Table S3 and Fig. S5, respectively.

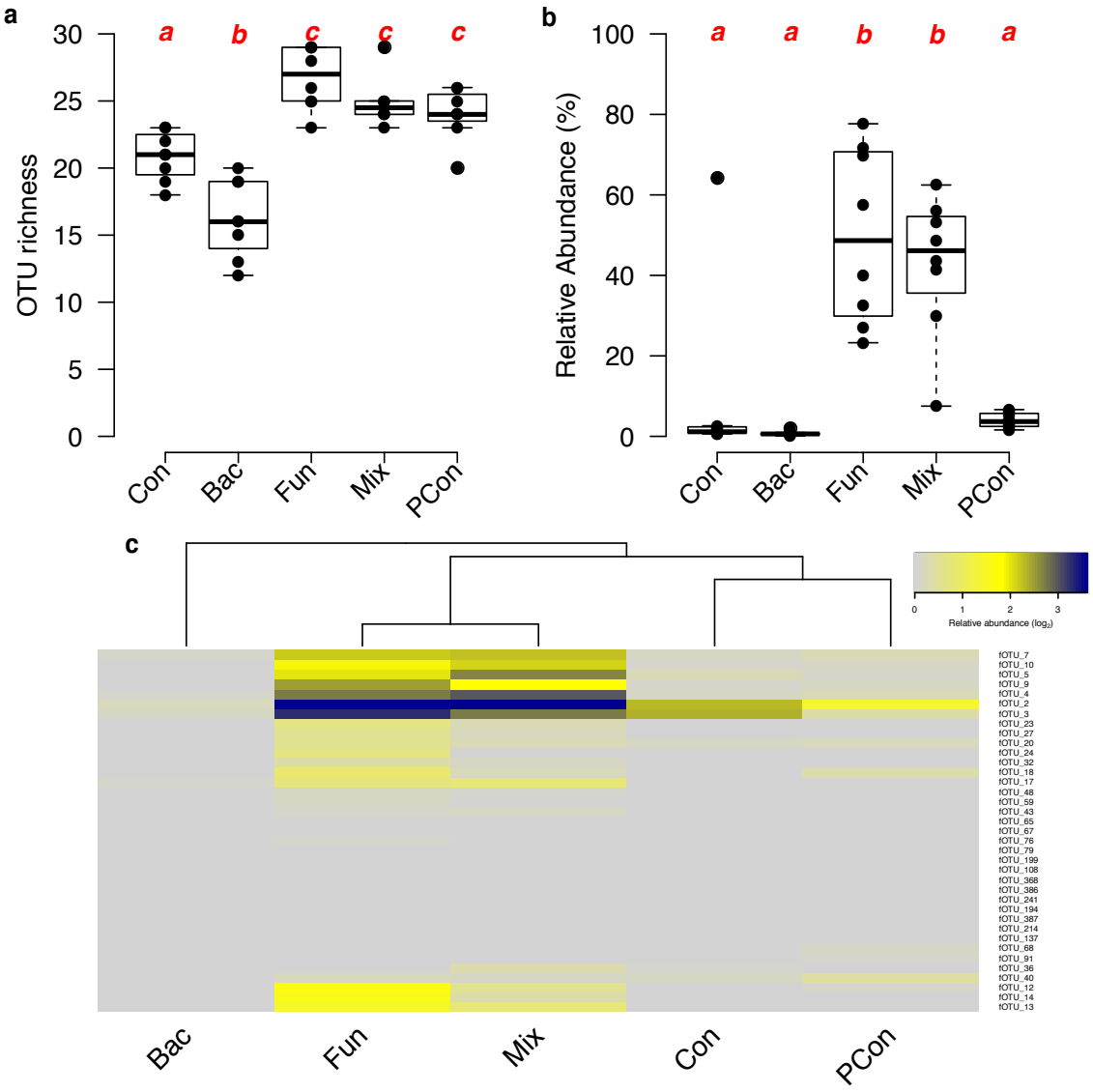

Figure 2: Establishment of fungi isolate-OTUs in the microcosms**. (a)** Number of inoculated fungi OTUs present in each microbial treatment. **(b)** Total relative abundance of inoculated OTU sequences in each microbial treatment expressed as percentage of total rarefied sequences. Different letters in **(a)** and **(b)** indicate significant differences between the different treatments as determined by ANOVA and a pairwise Tukey HSD test ($p<0.05$, Table S5). (c) Mean RA of the 33 inoculated OTUs present in the fungi dataset. Dendrogram reflects a clustering of the inoculated OTU community by Bray-Curtis dissimilarities with method "average." Raw data values and statistical testing for each OTU are present in Fig. S5.

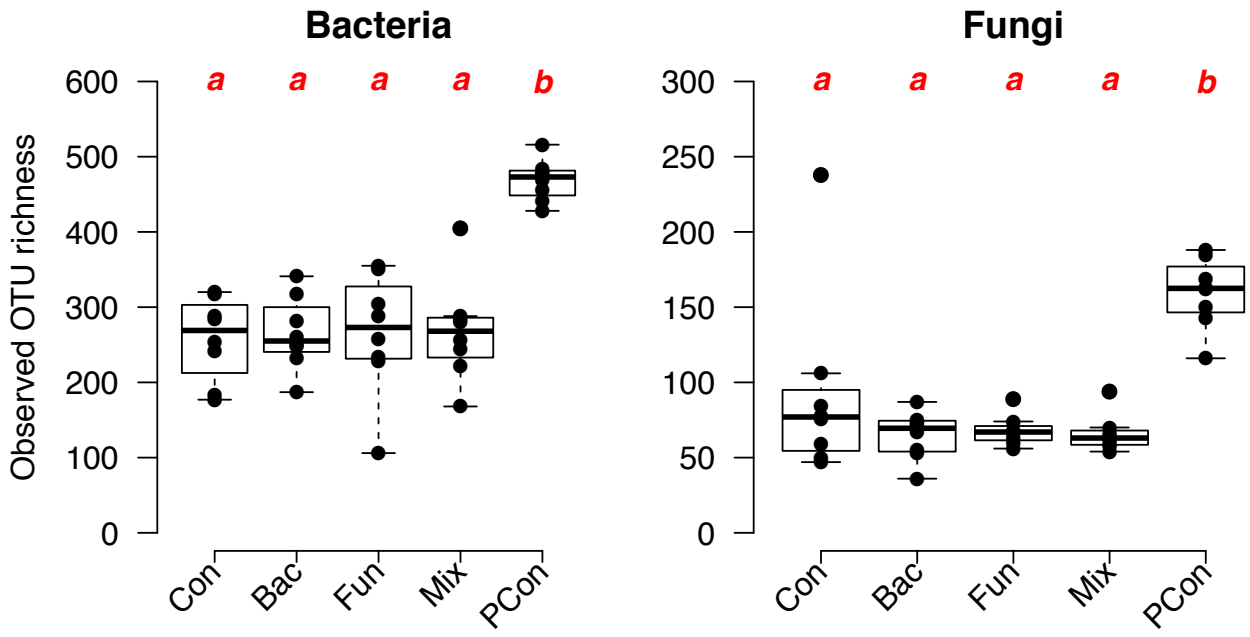

Figure 3: Total observed OTU richness for the complete bacteria and fungi communities across the five microbial treatments. OTU richness values are based on rarefying to 3,900 and 35,000 sequences per sample for the bacteria and fungi communities, respectively. Different letters indicate significant differences as determined by ANOVA and a Tukey post-hoc test ($p<0.05$, Table S2).

Total OTU richness in microcosm microbial communities

We tested whether inoculation of bacteria and fungi isolates into the autoclaved soil affected the total OTU richness in the different microbial treatments. For both the bacteria and fungi communities, the *PCon* treatment had a significantly higher OTU richness than the other four treatments (Fig. 3), being, on average, 1.8 times higher in bacteria community and 2.2 times higher in the fungi (Table S2). However, total bacteria and fungi OTU richness was similar across the other four treatments, and we found no significant differences between them (Fig. 3, Table S5). PCR testing whether DNA from freshly autoclaved soil (either from dead or live organisms) had survived sterilization, and thus potentially influenced out richness estimates, were inconclusive due to the presence of PCR inhibitors released during autoclaving (Fig. S6). However, plating dilutions of soil sampled from separately autoclaved microcosms placed in the greenhouse for 16 weeks revealed culturable bacteria and fungi colonies (Fig. S7), suggesting incomplete sterilization or contamination of the microcosms during the course of the experiment. However, the microbial load in the autoclaved soil was still noticeably lower (~1000x) compared to the non-autoclaved soil (Fig. S7).

Differences in microbial community composition

We determined the major components driving differences in the total bacteria and fungi communities in each microcosm using unconstrained ordination analysis. Differences between

the *PCon* treatment and the other treatments explained the most variation in the bacteria community (axis 1, 20.3% Fig. S8), and there was no distinct clustering among the other treatments. We observed considerably more variation within treatments in the fungi community, but sample clustering appeared to reflect the addition of the fungal inoculum, with the *Fun* and *Mix* treatments separated from the others (axis 1, 57.9%, Fig. S8).

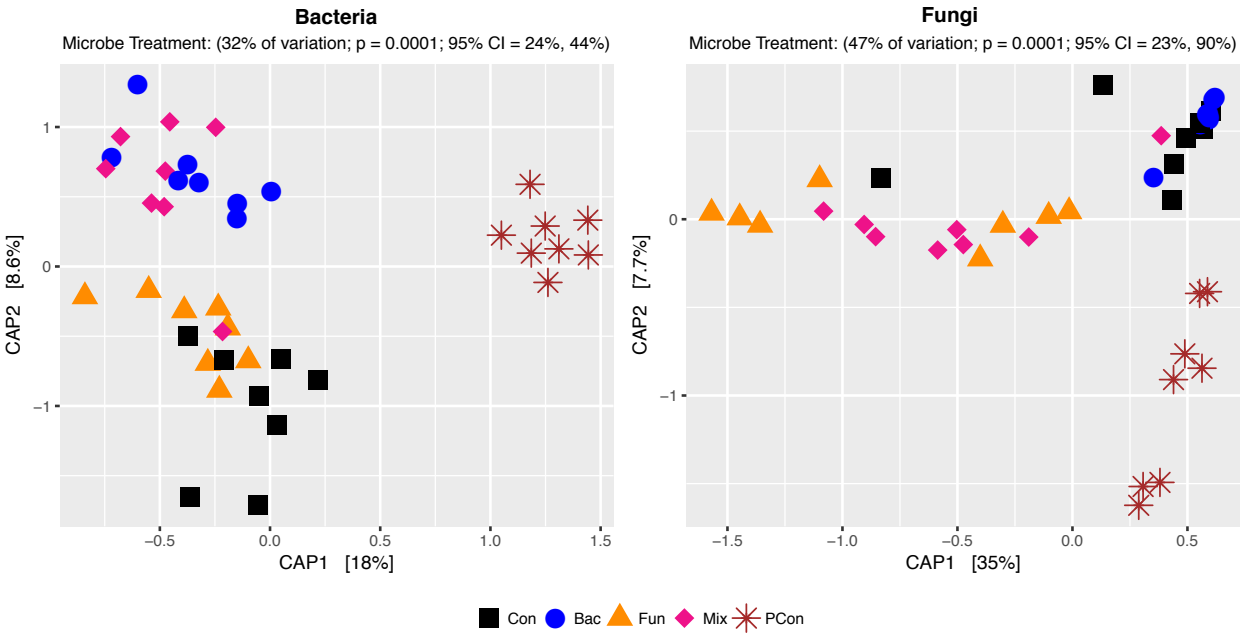

Figure 4: Constrained ordinations by microbial treatment of the entire bacteria and fungi dataset. Percentage of variation given on each axis refers to the explained fraction of total variation in each indicated community.

We then used constrained ordination and PERMANOVA to quantify the treatment effects on the microbial communities. Partial CAP – constrained by microbial treatment - highlighted differences between the *PCon* treatment and other treatments in the bacteria community, which explained 18% of the community variation. Separation of the samples on axis 2 reflected the addition of the bacteria inoculum, with the *Bac* and *Mix* treatments clustering together and away from the *Con* and *Fun* treatments. However, we noted that less variation was explained (Fig. 4). Conversely, in the fungi community, CAP axis 1 explained 35% of community variation and highlighted the effect of the fungal inoculation, with the *Fun* and *Mix* treatments clustering together and away from the others. Axis 2 explained considerably less variation and separated the *PCon* treatment from the others (Fig. 4). Differences between the microbial treatments in both communities were also statistically supported by PERMANOVA. In the bacteria community, we identified significant pairwise differences between all the treatments except for the *Bac* and *Mix*. In the fungi community, we

identified differences between the *PCon* and all other treatments, but no differences between the *Fun* and *Mix* (Table S6). A separate test for dispersion, which has also been shown to contribute to community differences [52], suggested that in both communities, differences between the *PCon* and other treatments were at least partially driven by dispersion. However, dispersion between the other treatments was largely the same, suggesting true biological differences between the communities (Table S6).

Microbial community effects on ecosystem functioning

The different microbial community treatments influenced some of the measured ecosystem functioning responses (Fig. 5, Table S7). Most notably, litter decomposition was, on average, 40% higher in the *PCon* treatment compared to the other treatments. Decomposition was generally greater in the *Fun* and *Mix* treatments compared to the *Bac* and *Con*, but the differences were not significant. Additionally, we noted that net primary productivity in the *Mix* treatment was significantly lower (-14%) compared to the *Con* treatment. Leaching volumes were generally consistent across all treatments and were unaffected by the microbial treatment. We also found no significant effect of the microbial treatment on $N\text{-}N_2O$ losses. We explored whether certain fungi isolates contributed to the measured ecosystem functions by correlating the RAs of the 22 inoculated fungi OTUs found to be significantly more abundant in *Fun* and *Mix* treatments (Fig. 2c, Fig. S4) and the ecosystem functioning responses. However, this analysis revealed no significant correlations (Table S8).

Lastly, we assessed the effects of the microbial community treatments on ecosystem multifunctionality by calculating a multifunctionality index for each microcosm. This was performed by averaging z-transformed data values of the investigated ecosystem functions (see Materials and Methods). Overall, we found ecosystem multifunctionality responses were lower in the *Mix* treatment compared to the *Con* and *PCon* treatments (Fig. 6, Table S7).

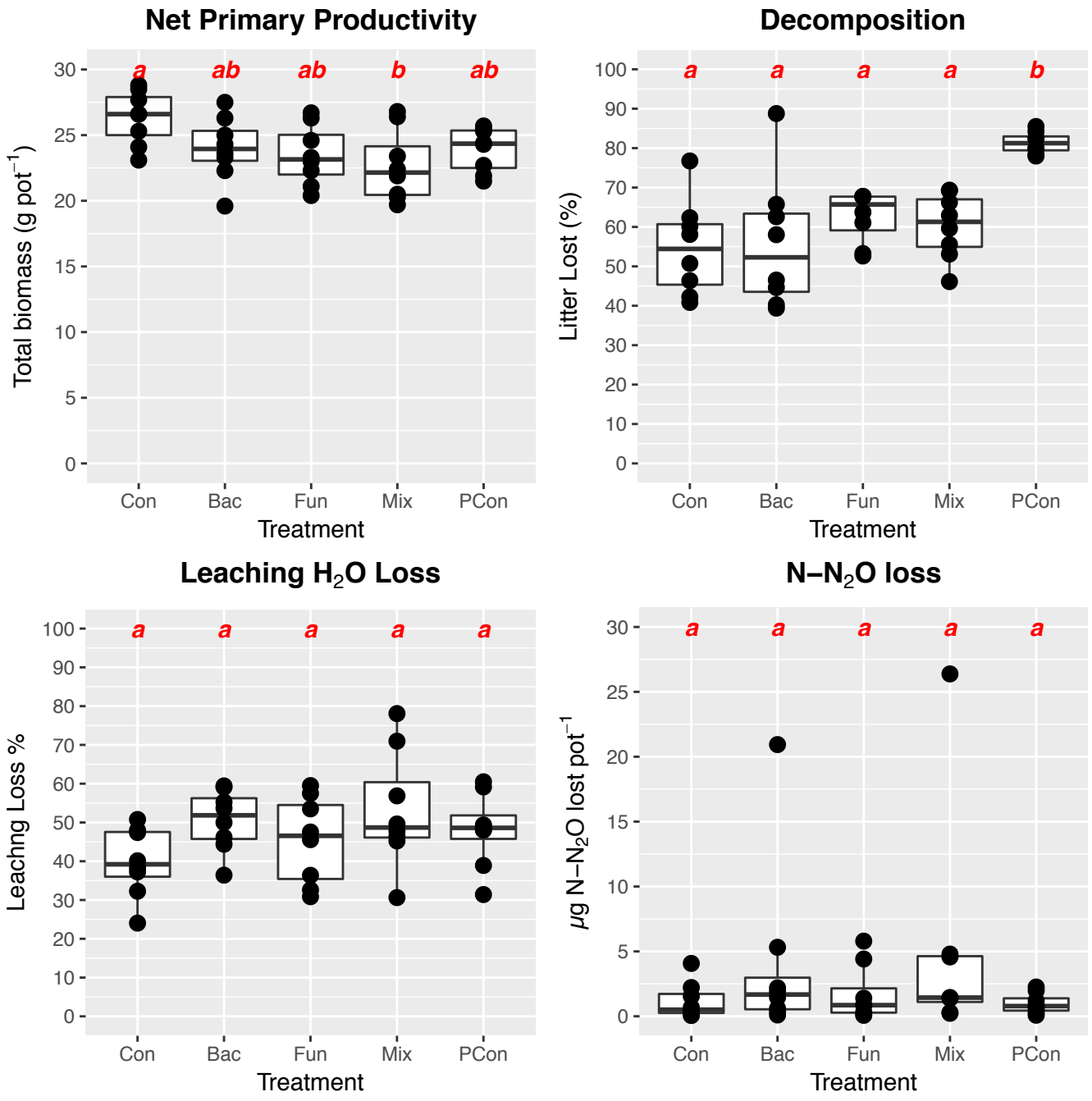

Figure 5: Responses of the measured ecosystem functions to the microbial treatments. Letters indicate significant differences between the treatments as determined using a Tukey HSD test ($p<0.05$).

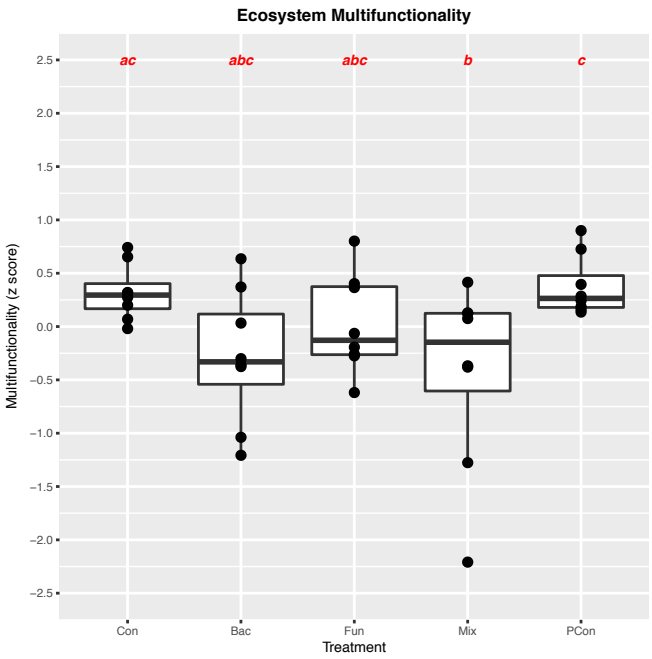

Figure 6: Experimental microbial treatment effects on total ecosystem functionality. Means of z-transformed values for the individual ecosystem functions were calculated to create a multifunctionality value for each microcosm. Different letters indicate significant differences between the treatments as determined using a Tukey HSD test ($p<0.05$).

Discussion

We have used reference stocks of bacteria and fungi isolates to inoculate specially designed microcosms and investigated the effects of manipulating soil bacteria and fungi communities on specific ecosystem functions and overall ecosystem multifunctionality. Previous studies have experimentally investigated the link between soil microbial diversity and ecosystem functioning using a similar microcosm system and successfully manipulated general soil biodiversity through the use of progressively smaller sieve sizes [19,25] or manipulated the presence of specific groups of microbes like AMF and rhizobia [18]. Here, we aimed to expand on these efforts and manipulate general soil bacteria and fungi dominance by means of individual isolate inoculation into autoclaved soil. We have shown that some of the inoculated bacteria and fungi were able to establish inside the microcosms, but this did not result in significant differences in total bacteria or fungi richness between the microbial community treatments, with the exception of the *PCon* treatment. However, we found differences in soil bacteria and fungi community composition, suggesting microbe inoculation resulted in quantitative, rather than qualitative, changes in the soil communities. While we found greater litter decomposition in the *PCon* treatment, effects of the microbial treatments on individual and general ecosystem functioning were subtle, but our results show that the community composition of the *Mix* treatment reduced plant biomass production and overall ecosystem

functioning. Overall, this study demonstrates that we still face still considerable challenges when trying to manipulate general soil microbial diversity in experimental microcosm systems and assess the effects on ecosystem functioning and serves as a starting point for future experiments.

Detection of inoculated isolates in the microcosms

We utilized amplicon sequencing to characterize the composition of endpoint microbial community in each microcosm and mapped, based on sequence similarity, the sequences of the inoculated isolates to the reference OTUs from each kingdom's community profile. With this, we aimed to determine which of the inoculated bacteria and fungi strains were able to establish inside the microcosms. In both the bacteria and fungi communities, we found that well over 50% of the inoculated isolates clustered to an OTU in their respective kingdoms, with some community profile OTUs being represented by multiple isolate sequences. We attribute this to the fact that we clustered the isolate sequences into OTUs for inoculum creation based on the full length Sanger sequences and subsequently trimmed them in order to compare the same operon used for community profiling, thereby reducing the resolution at which different OTUs could be differentiated. This could be addressed in future experiments in two ways. First, for inoculum creation, isolate sequences could be initially trimmed to identify the same sub-region of the gene fragment to be used for end-point community profiling and then clustered into OTUs. This would reduce OTU richness in the inoculum but allow 1 to 1 agreement when clustering the isolate sequences to community reference OTUs. Second, recent advances in sequencing technology (e.g. Pacific Biosciences (PacBio) Single Molecule, Real Time DNA Sequencing System) have made it possible to sequence nearly full-length gene fragments at error rates comparable to other sequencing platforms [53]. In our case, the use of the PacBio system for community profiling would permit the mapping of the full-length isolate sequences to the full-length sequences of the community reference OTUs and provide greater resolution to detect the isolates that were present in the microcosms. Although such single-molecule sequencing technologies typically trade read length for the number of reads per sample [53], we would expect such a trade-off to have negligible effects on diversity estimates in our system given the presumably low initial OTU richness of our microbial inocula.

Establishment of inoculated isolates

We did not detect sequences for 15 of the 48 inoculated bacteria (Table S2) and 6 of the 45 inoculated fungi isolates in the community profiles (Table S3). Moreover, none of the isolates with a matching community profile OTU in the bacteria community had a significantly

higher RA in the inoculated treatments and some had a very low overall RA (Fig. 3c, Fig. S4, Table S2). This suggests that all isolates in the bacteria community, and some of the isolates in the fungi community failed to establish in the microcosms. One likely explanation is that physicochemical differences between the sand/soil substrate in the microcosms and native soil exerted selective pressure on the inoculated microbes and favored those that could quickly adapt to the new growth conditions, as the diversity patterns of soil microbes are strongly influenced by edaphic factors and their interactions with other microorganisms [54,55]. A second explanation refers to the source of the reference stock isolates. We chose to use bacteria and fungi isolated from living Trifolium roots because they were a readily available source of microbial inoculants. However, evidence suggests that root microbes undergo some level of co-adaptation to their host plant [56] as a result of the specialized conditions created in the rhizosphere through the rhizodeposition of an array of carbon and antimicrobial compounds and signaling hormones [57]. Moreover, as root microbial communities have been shown to differ between plant species [58,59], some of the inoculated strains may have been specially adapted to Trifolium roots. Therefore, inoculating root isolates into bulk soil planted with a grass species likely exerted further selective pressure on the bacteria and fungi communities. These shortcomings could be addressed in the future, particularly when more general soil microbial functions are being investigated, by conducting large scale isolation efforts from the experimental soil and subsequently utilizing these soil-adapted isolates in the inoculation experiments.

Assessing bacteria and fungi dominance

By inoculating the different bacteria and fungi isolates, we aimed to create treatments dominated by bacteria, fungi, or a combination of both. However, our use of amplicon-based community sequencing only permitted the comparison of differences in bacteria and fungi richness and taxa relative abundances between the treatments. Thus, here we have equated the term “dominance” with significantly higher bacteria or fungi richness. We found isolate inoculation did not result in differences in total bacteria or fungi richness (Fig. 3), but we cannot exclude the possibility that inoculation resulted in differences in the absolute abundances of bacteria or fungi because our community profiling method does not allow for absolute quantification of the total bacteria and fungi abundance in each community. Answering such questions requires different molecular tools, such as quantitative real-time PCR (qPCR), which can be used to quantify the number of 16S or ITS gene copies in a sample to shed light on total bacteria and fungi community size, respectively. Therefore, qPCR, should be utilized in future

experiments to complement community profiling data and to more effectively answer the question if total bacteria and fungi abundance in soils can be manipulated by inoculation.

Possible contamination masks effects of microbial inoculation on total richness and community composition

In addition to no differences in total bacteria and fungi richness between the different treatments (with the exception of the *PCon* treatment), we also unexpectedly detected inoculated bacteria and fungi OTUs in treatments that received no microbial inoculum (Figs. 1a, 2a). These results are likely due to one or a combination of a number of reasons. First, it remains possible that the microcosm soil was not completely sterile at the start of the experiment. Although our plating of autoclaved and non-autoclaved soil samples suggested no viable bacteria or fungi remained after autoclaving (Fig. S7), this conclusion is limited by our use of a single culture medium and the fact that many microbes cannot be cultured [60].

Second, we cannot exclude the possibility that the presence of inoculated OTUs or our total richness and diversity estimates were influenced by surviving DNA from dead or sterilization resistant microbes. We attempted to test for this by performing PCR on DNA extracted from autoclaved soil samples, but the presence of PCR inhibitors in these autoclaved samples means we cannot definitely conclude this occurred in our case (Fig. S6). However, more conclusive evidence for sterilization resistant DNA was found in the fungi community, where we detected a high RA of a single OTU classified as the AMF species *Rhizophagus irregularis* (data not shown). Interestingly, visual microscopic examination of stained roots revealed no AMF colonization, suggesting there were no viable AMF in the soil (Alain Held, personal communication). PCR is notoriously poor at differentiating between DNA from live, viable cells and free DNA from dead ones, which can adsorb to soil particles [61,62]. Moreover, it was recently shown that DNA from dead microbes represents a large fraction of the DNA in soil and can result in inflated prokaryotic and fungi diversity and the misestimation of taxon abundances [63]. However, it has been demonstrated that distinct microbial communities in different soils can still be discriminated between even when inactive DNA is not removed [63]. This supports our conclusion from the ordination and PERMANOVA analyses that microbial inoculation at least partly induced changes in overall community composition (Fig. 4, Table S6). Future experiments would benefit from removing DNA from inactivated cells to better estimate richness and focus on the active microbial community. This could be accomplished through the use of treating the soil samples collected at the end of the experiment with propidium monoazide (PMA), a photo-activated dye that permeates

compromised cell membranes, which are considered to be non-viable, and binds with DNA, resulting in strong inhibition of PCR amplification [61,64]. Treating soil samples with PMA before DNA extraction and PCR was recently shown to remove DNA from dead microbes that caused overestimation of bacteria and fungi diversity in a wide variety of soils [63]. Traditionally, RNA based sequencing approaches have been thought to differentiate between metabolically active and inactive cells and have been used to describe both active soil bacteria [65] and fungi [65,66] communities. However, recent work has suggested using RNA analysis to characterize active microbes is not valid in many circumstances [67]. Furthermore, given the financial and time costs of RNA extraction and sequencing, it may be more cost effective to treat samples with PMA to address the issue of characterizing only the viable microbe community [63].

Third, while we set up the experiment under a sterile bench and used air and water filters to prevent contamination, the microcosms may have become contaminated during the course of the experiment either by outside microbes or seed endophytes. Contamination of the microcosms by outside microbes is supported by our finding of culturable bacteria and fungi after separately autoclaved microcosms were allowed to sit, sealed, in the greenhouse for 16 weeks (Fig. S7). A number of recent studies have utilized artificial growth substrates like calcined clay [27,30,68] or vermiculite [69] in combination with microcosm systems to successfully investigate microbial contributions to plant functioning. Thus outside contamination in our system could potentially be addressed in the future through the use of such artificial growth substrates which contain a smaller native microbial community, and thus may be easier to autoclave and keep sterile.

Finally, contaminating bacteria and fungi could have unintentionally been introduced during the experimental set up due to contaminated start inoculum, possibly the result of impure reference stock strains or other environmental contamination. This highlights the need for careful handling of the isolated strains and verification of their purity (i.e. via multiple rounds of sub culturing on a variety of media containing antibiotics or fungicides and/or cross kingdom PCR). Additionally, samples of the starting microbial inoculum should be included in future sequencing runs. Not only would this allow for the identification of potential contamination, but also the quantification of the change between the input and final microbial community [27] and more precise identification of inoculum-derived OTUs versus those natively present in soil.

Despite the shortcomings discussed above that potentially influenced our richness and diversity estimates, we found that in the inoculated fungi community the richness and total RA

of inoculated OTUs was significantly higher in treatments receiving the fungal inoculum (Fig. 2a,b), whereas this was not the case in the inoculated bacteria community (Fig. 1a,b). These results confirm that despite some fungal contamination, possibly due to the reasons mentioned above, we appeared to be more successful in manipulating the inoculated soil fungi community. This conclusion is further supported by the fact that 22 of the inoculated fungi OTUs were significantly more abundant in the *Fun* and/or *Mix* treatments compared to the non-inoculated treatments (Fig. S5), whereas we found no significant differences in the RAs of any of the inoculated bacteria OTUs (Fig. S4). Thus, our results present an encouraging example that the abundance of certain fungi isolates can be successfully manipulated in the microcosms. Therefore, when combined with the suggestions for improvement outlined above, future work may benefit from focusing on the manipulation and characterization of the active soil fungi community in the microcosms and its effects on ecosystem functioning.

Microbial community effects on ecosystem functioning and multifunctionality

Effects of the different microbial treatments on the individual ecosystem functions measured were limited to litter decomposition and biomass production (Fig. 5). We found no significant differences in decomposition between the treatments receiving the prepared microbial inocula, suggesting the inocula did not contain active saprotrophs. This could be because the reference stock bacteria and fungi were isolated from living plant roots. However, our finding that litter decomposition was significantly higher in the *PCon* treatment, which had the highest bacteria and fungi richness, is consistent with previous work utilizing similar microcosm systems [19,25]. Positive effects of microbial richness and diversity on litter decomposition are thought to be the result of a combination of facilitative interactions and resource partitioning between microbes which results in a wider range of plant polymers being degraded and thus higher rates of decomposition [70]. In addition to greater microbial richness, higher litter decomposition in the *PCon* treatment could also be the result of the presence of mesofauna, which also play important roles in mediating litter decomposition in terrestrial ecosystems [71]. Although we sieved the soil, removing soil macrofauna like earthworms and larger soil insects, the soil inoculated in the *PCon* treatment likely contained smaller soil animals like nematodes, collembola, and mites, whose abundances in a single gram of soil can be in the thousands [5]. However, the presence of a mesofauna community should be confirmed and characterized in future work. Additionally, it may also be interesting to include DNA extracted from remaining plant litter in sequencing runs to explore if specific bacteria or fungi

taxa are enriched in litter samples, potentially revealing the importance of these taxa in decomposition processes.

Our results showing significantly lower biomass production in the *Mix* treatment compared to the *Con* treatment is probably best explained by differences in the composition of the soil bacteria and fungi communities in each treatment (Fig. 4, Table S6). The composition of this soil microbe community is the most influential factor determining the composition a plant's root-associated microbe community [72,73], which interacts more directly with the plant and therefore plays an important role in determining plant growth and development [9,74]. Therefore, it may be worthwhile to also characterize root-associated microbe communities in future experiments to better investigate the link between microbe community richness and composition and plant biomass production.

Combining the individual ecosystem functions into one multifunctionality index revealed lower overall ecosystem functioning in the *Mix* treatment compared to the *Con* and *PCon* treatments, but this appeared to be the result of one replicate where N losses were very high (Fig. 6). We chose not to remove this replicate from the analysis because we found no evidence that the richness or microbial community composition in this microcosm presented an outlier. Thus, large variations in ecosystem functioning can exist between replicates even when their microbial communities are largely similar. Furthermore, while our choice to average the individual ecosystem functions into a single multifunctionality value has been used previously [19,51], our results highlight that single functions can have a large impact on estimates of overall ecosystem multifunctionality [75]. Consequently, future work may assess more individual ecosystem functions like nutrient losses from leaching or nutrient turnover and subsequently explore newer, alternative methods of calculating ecosystem multifunctionality [75].

Final thoughts

We have reported our successes and challenges in manipulating bacteria and fungi communities in microcosm systems and assessing the effects on ecosystem functioning. Despite the shortcomings we have discussed with regard to potential contamination and the possible effects of DNA from dead microbes, our results suggest that a number of fungi isolates were able to establish inside the microcosms. Moreover, differences in total bacteria and fungi community composition likely resulted in changes in litter decomposition and plant productivity. These results present an encouraging example of how reference stocks of microbial isolates and microcosm systems can be combined to elucidate general microbial

contributions to certain ecosystem functions. By implementing the suggestions for improvement discussed here, we think such systems can become an even more a valuable tool for exploring ecosystem functioning responses to changing bacteria and fungi communities.

Acknowledgements

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References

1. Cardinale BJ, Duffy JE, Gonzalez A, Hooper DU, Perrings C, Venail P, et al. Biodiversity loss and its impact on humanity. Nature. 2012;489:326–326.

2. Hooper D, Chapin III F, Ewel J, Hector A, Inchausti P, Lavorel S, et al. Effects of Biodiversity on Ecosystem Functioning: A Consensus of Current Knowledge. Ecol. Monogr. 2005;75:3–35.

3. Hector A, Bagchi R. Biodiversity and ecosystem multifunctionality. Nature. 2007;448:188–90.

4. Loreau M, Naeem S, Inchausti P, Bengtsson J, Grime JP, Hector A, et al. Biodiversity and ecosystem functioning: current knowledge and future challenges. Science. 2001;294:804–8.

5. Bardgett RD, van der Putten WH. Belowground biodiversity and ecosystem functioning. Nature. 2014;515:505–11.

6. Brussaard L, Behan-Pelletier V, Bignell D, Brown V, Didden W, Folgarait P, et al. Biodiversity and ecosystem functioning in soil. Ambio. 1997;26:563–70.

7. van der Heijden MGA, Bardgett RD, Van Straalen NM. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol. Lett. 2008;11:296–310.

8. Six J, Frey SD, Thiet RK, Batten KM. Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems. Soil Sci. Soc. Am. J. 2006;70:555.

9. Wardle DA, Bardgett RD, Klironomos JN, Setälä H, van der Putten WH, Wall DH. Ecological linkages between aboveground and belowground biota. Science. 2004;304:1629–33.

10. Strickland MS, Rousk J. Considering fungal:bacterial dominance in soils – Methods, controls, and ecosystem implications. Soil Biol. Biochem. 2010;42:1385–95.

11. Helgason BL, Walley FL, Germida JJ. Fungal and bacterial abundance in long-term no-till and intensive-till soils of the Northern Great Plains. Soil Sci. Soc. Am. J. 2009;73:120–7.

12. van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, et al. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature. 1998;396:69–72.

13. Wagg C, Jansa J, Stadler M, Schmid B, van der Heijden MGA. Mycorrhizal fungal identity and diversity relaxes plant-plant competition. Ecology. 2011;92:1303–13.

14. Bender SF, Plantenga F, Neftel A, Jocher M, Oberholzer H-R, Köhl L, et al. Symbiotic relationships between soil fungi and plants reduce N_2O emissions from soil. ISME J. 2013;1–10.

15. Wagg C, Jansa J, Schmid B, van der Heijden MGA. Belowground biodiversity effects of plant symbionts support aboveground productivity. Ecol. Lett. 2011;14:1001–9.

16. van der Heijden MGA, Bakker R, Verwaal J, Scheublin TR, Rutten M, van Logtestijn R, et al. Symbiotic bacteria as a determinant of plant community structure and plant productivity in dune grassland. FEMS Microbiol. Ecol. 2006;56:178–87.

17. Setälä H, McLean MA. Decomposition rate of organic substrates in relation to the species diversity of soil saprophytic fungi. Oecologia. 2004;139:98–107.

18. van der Heijden MG, de Bruin S, Luckerhoff L, van Logtestijn RS, Schlaeppi K. A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity, plant nutrition and seedling recruitment. ISME J. 2016;10:389–99.

19. Wagg C, Bender SF, Widmer F, van der Heijden MGA. Soil biodiversity and soil community composition determine ecosystem multifunctionality. Proc. Natl. Acad. Sci. U. S. A. 2014;1–5.

20. Schnitzer SA, Klironomos JN, Hille Ris Lambers J, Kinkel LL, Reich PB, Xiao K, et al. Soil microbes drive the classic plant diversity-productivity pattern. Ecology. 2011;92:296–303.

21. Chaparro JM, Sheflin AM, Manter DK, Vivanco JM. Manipulating the soil microbiome to increase soil health and plant fertility. Biol. Fertil. Soils. 2012;48:489–99.

22. Griffiths BS, Ritz K, Bardgett RD, Cook R, Christensen S, Ekelund F, et al. Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions : an examination of the biodiversity – ecosystem function relationship. Oikos. 2000;2:279–94.

23. Wertz S, Degrange V, Prosser JI, Poly F, Commeaux C, Freitag T, et al. Maintenance of soil functioning following erosion of microbial diversity. Environ. Microbiol. 2006;8:2162–9.

24. Wertz S, Degrange V, Prosser JI, Poly F, Commeaux C, Guillaumaud N, et al. Decline of soil microbial diversity does not influence the resistance and resilience of key soil microbial functional groups following a model disturbance. Environ. Microbiol. 2007;9:2211–9.

25. Pellkofer S. Soil Biodiversity and Ecosystem Functioning. University of Zürich; 2015.

26. Schlaeppi K, Bulgarelli D. The Plant Microbiome at Work. Mol. Plant Microbe Interact. 2015;28:212–7.

27. Bai Y, Müller DB, Srinivas G, Garrido-Oter R, Potthoff E, Rott M, et al. Functional overlap of the Arabidopsis leaf and root microbiota. Nature. 2015;528:364–9.

28. Lebeis SL, Paredes SH, Lundberg DS, Glavina T, Jones CD. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. Science. 2015;349:1678–81.

29. Wittwer RA, Dorn B, Jossi W, van der Heijden MGA. Cover crops support ecological intensification of arable cropping systems. Sci. Rep. 2017;7:10.1038/srep41911.

30. Hartman K, van der Heijden MG, Roussely-Provent V, Walser J-C, Schlaeppi K. Deciphering composition and function of the root microbiome of a legume plant. Microbiome. 2017;5:10.1186/s40168-016-0220-z.

31. Freeman S, Katan T. Identification of colletotrichum species responsible for anthracnose and root necrosis of strawberry in Israel. Phytopathology. 1997;87:516–21.

32. White T, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: Innis M, Gelfland D, Sninsky J, White T, editors. PCR Protoc. A Guid. to Methods Appl. San Diego, CA: Academic Press; 1990. p. 315–22.

33. Rice P, Longden I, Bleasby A. EMBOSS: The European Molecular Biology Open Software Suite. Trends Genet. 2000;16:276–7.

34. Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets. Bioinformatics. 2011;27:863–4.

35. Abarenkov K, Henrik Nilsson R, Larsson K-H, Alexander I, Eberhardt U, Erland S, et al. The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytol. 2010;186:281–5.

36. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 2010;7:335–6.

37. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26:2460–1.

38. Lane D. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid Tech. Bact. Syst. New York, NY: John Wiley and Sons; 1991. p. 115–75.

39. Coombs JT, Franco CMM. Isolation and Identification of Actinobacteria from Surface-Sterilized Wheat Roots. Appl. Environ. Microbiol. 2003;69:5603–8.

40. Chelius MK, Triplett EW. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. 2001;41:252–63.

41. Bodenhausen N, Horton MW, Bergelson J. Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. PLoS One. 2013;8:e56329.

42. Faircloth BC, Glenn TC. Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. PLoS One. 2012;7:e42543.

43. Gardes M, Bruns T. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 1993;2:113–8.

44. Dodt M, Roehr JT, Ahmed R, Dieterich C. FLEXBAR-Flexible Barcode and Adapter Processing for Next-Generation Sequencing Platforms. Biology (Basel). 2012;1:895–905.

45. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2015.

46. Weiss SJ, Xu Z, Amir A, Peddada S, Bittinger K, Gonzalez A, et al. Effects of library size variance, sparsity, and compositionality on the analysis of micrbiome data. PeerJ Prepr. 2015;

47. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. vegan: Community Ecology Package. 2015.

48. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One. 2013;8:e61217.

49. Hervé M. RVAideMemoire: Diverse Basic Statistical and Graphical Functions. 2016.

50. Jurasinkski G, Koebsch F, Guenther A, Beetz S. flux: Flux rate calculation from dynamic closed chamber measurements. 2014.

51. Maestre FT, Quero JL, Gotelli NJ, Escudero A, Ochoa V, Delgado-Baquerizo M, et al. Plant Species Richness and Ecosystem Multifunctionality in Global Drylands. Science. 2012;335:214–8.

52. Anderson MJ. A new method for non parametric multivariate analysis of variance. Austral Ecol. 2001;26:32–46.

53. Schloss PD, Jenior ML, Koumpouras CC, Westcott SL, Highlander SK. Sequencing 16S rRNA gene fragments using the PacBio SMRT DNA sequencing system. PeerJ. 2016;4:e1869.

54. Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G. Microbial diversity and soil functions. Eur. J. Soil Sci. 2003;54:655–70.

55. Fierer N, Jackson RB. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. U. S. A. 2006;103:626–31.

56. Bakker MG, Manter DK, Sheflin AM, Weir TL, Vivanco JM. Harnessing the rhizosphere microbiome through plant breeding and agricultural management. Plant Soil. 2012;360:1–13.

57. Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A. The importance of the microbiome of the plant holobiont. New Phytol. 2015;206:1196–206.

58. Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH. Going back to the roots: the microbial ecology of the rhizosphere. Nat. Rev. Microbiol. 2013;11:789–99.

59. Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 2013;64:807–38.

60. Hugenholtz P, Pace NR. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. Trends Biotechnol. 1996;14:190–7.

61. Cangelosi GA, Meschke JS. Dead or alive: Molecular assessment of microbial viability. Appl. Environ. Microbiol. 2014;80:5884–91.

62. Taberlet P, Prud'Homme SM, Campione E, Roy J, Miquel C, Shehzad W, et al. Soil sampling and isolation of extracellular DNA from large amount of starting material suitable for metabarcoding studies. Mol. Ecol. 2012;21:1816–20.

63. Carini P, Marsden PJ, Leff JW, Morgan EE, Strickland MS, Fierer N. Relic DNA is abundant in soil and obscures estimates of soil microbial diversity. Nat. Microbiol. 2016;2:16242.

64. Nocker A, Sossa-Fernandez P, Burr MD, Camper AK. Use of propidium monoazide for live/dead distinction in microbial ecology. Appl. Environ. Microbiol. 2007;73:5111–7.

65. Baldrian P, Kolařík M, Štursová M, Kopecký J, Valášková V, Větrovský T, et al. Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. ISME J. 2012;6:248–58.

66. Rajala T, Peltoniemi M, Hantula J, Mäkipää R, Pennanen T. RNA reveals a succession of active fungi during the decay of Norway spruce logs. Fungal Ecol. 2011;4:437–48.

67. Blazewicz SJ, Barnard RL, Daly RA, Firestone MK. Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. ISME J. 2013;7:2061–8.

68. Lebeis SL, Paredes SH, Lundberg DS, Breakfield N, Gehring J, McDonald M, et al. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. Science. 2015;349:860–4.

69. Haney CH, Samuel BS, Bush J, Ausubel FM. Associations with rhizosphere bacteria can confer an adaptive advantage to plants. Nat. Plants. 2015;1:1–9.

70. Gessner MO, Swan CM, Dang CK, McKie BG, Bardgett RD, Wall DH, et al. Diversity meets decomposition. Trends Ecol. Evol. 2010;25:372–80.

71. Hättenschwiler S, Tiunov A V., Scheu S. Biodiversity and Litter Decomposition in Terrestrial Ecosystems. Annu. Rev. Ecol. Evol. Syst. 2005;36:191–218.

72. Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, et al. Defining the core Arabidopsis thaliana root microbiome. Nature. 2012;488:86–90.

73. Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, et al. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature. 2012;488:91–5.

74. Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and plant health. Trends Plant Sci. 2012;17:478–86.

75. Byrnes JEK, Gamfeldt L, Isbell F, Lefcheck JS, Griffin JN, Hector A, et al. Investigating the relationship between biodiversity and ecosystem multifunctionality: Challenges and solutions. Methods Ecol. Evol. 2014;5:111–24.

Supplementary Information

Ecosystem functioning under bacteria and fungi dominated soil communities

Supplementary Figures

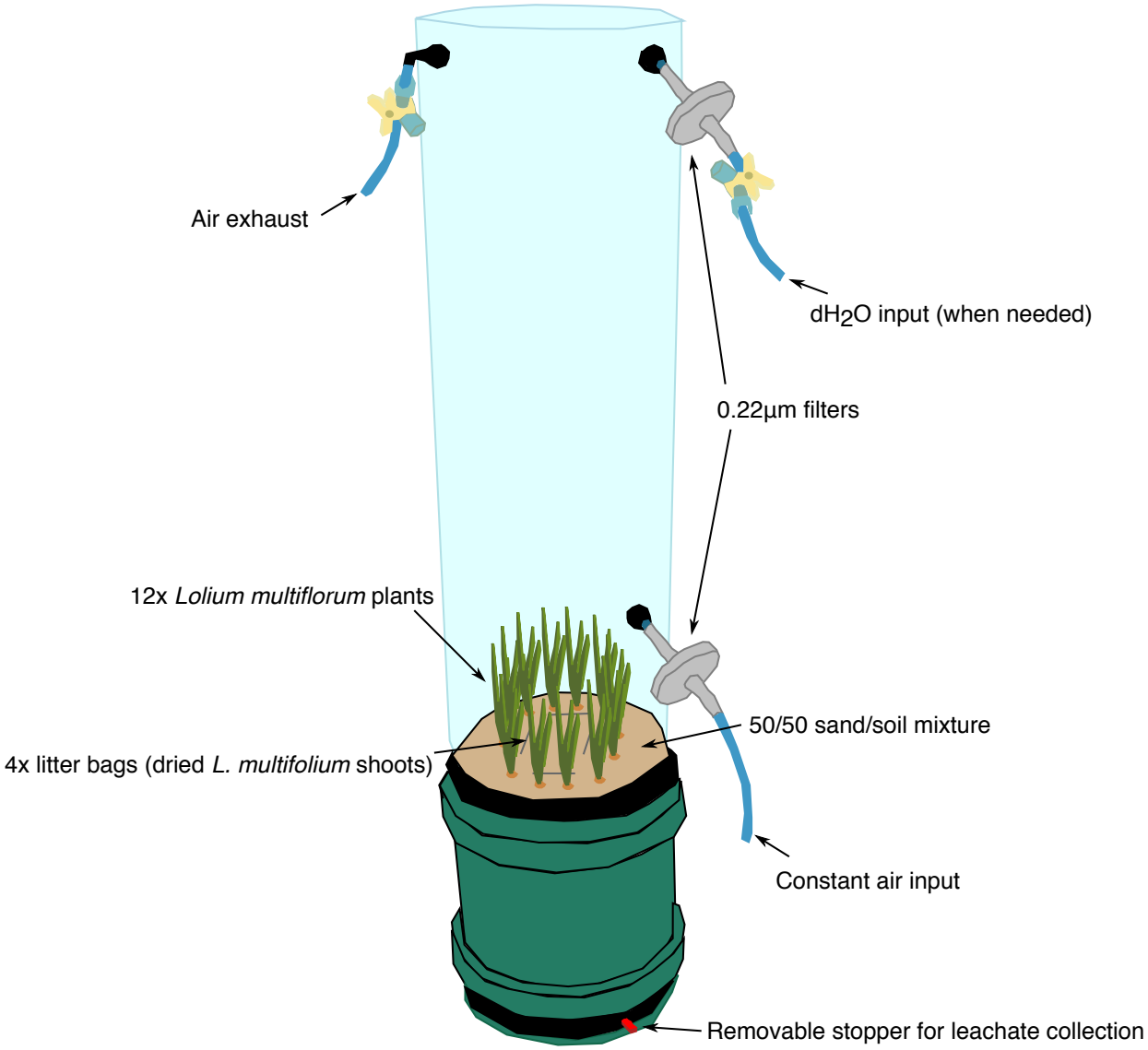

Figure S1: Schematic drawing the experimental microcosms. Sand/soil mixture is added to the bottom pot, litter bags buried in the soil, and plants planted. The pots are closed with the clear plastic lid and incoming air and water passed through sterile filters. The microcosms contain an air exhaust outlet for gas measurements and a removable stopper for leachate collection.

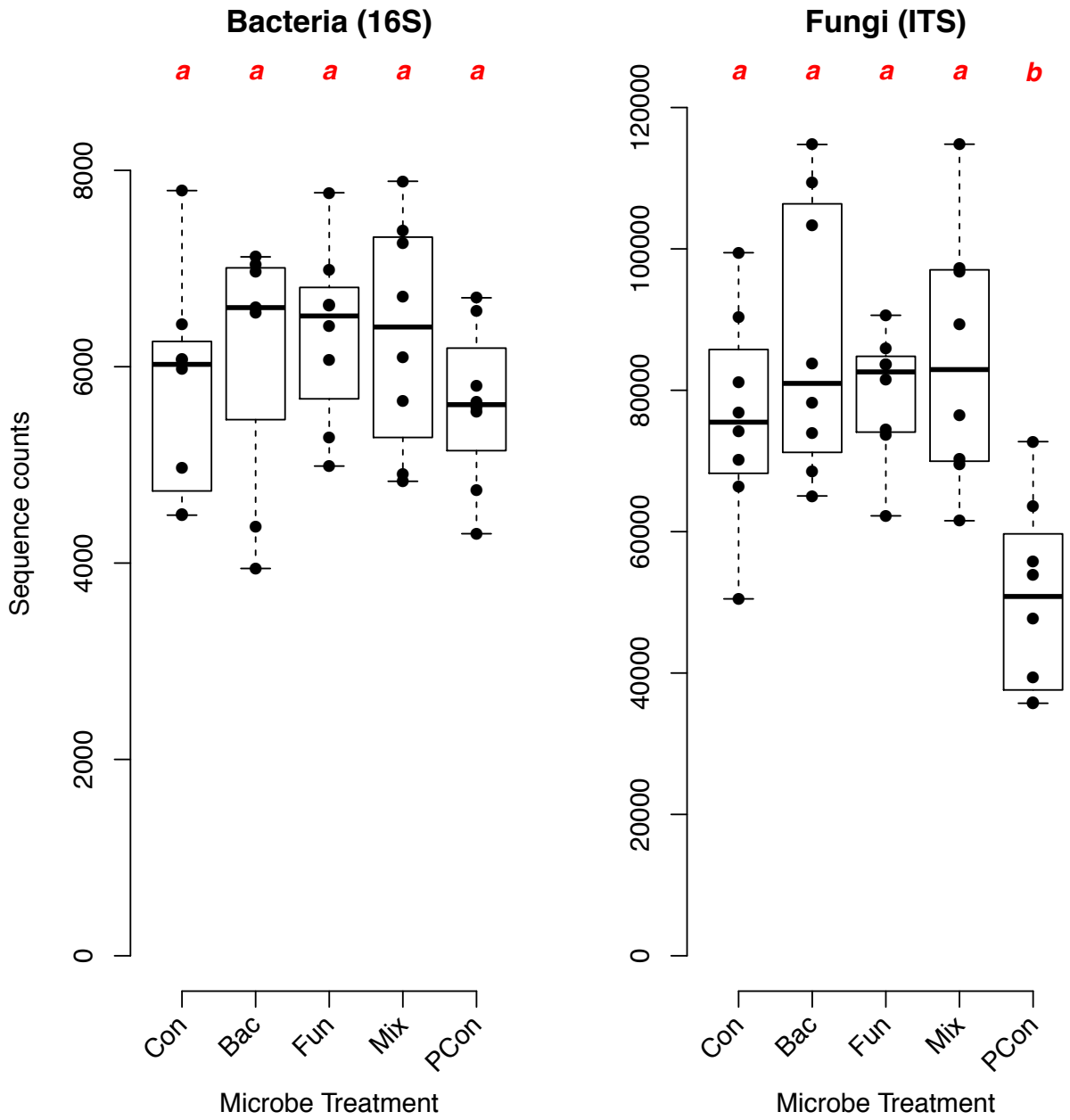

Figure S2: Distribution of 16S and ITS amplicon sequence counts for the experimental microbial treatments. Letters indicate significant differences between the groups according to a pairwise Wilcox test ($p<0.05$, FDR corrected). Significant differences in the ITS sequencing depth resulted in rarefaction of the dataset to 35,000 and 3,900 sequences per sample for the ITS and 16S communities, respectively. See Materials and Methods for more information.

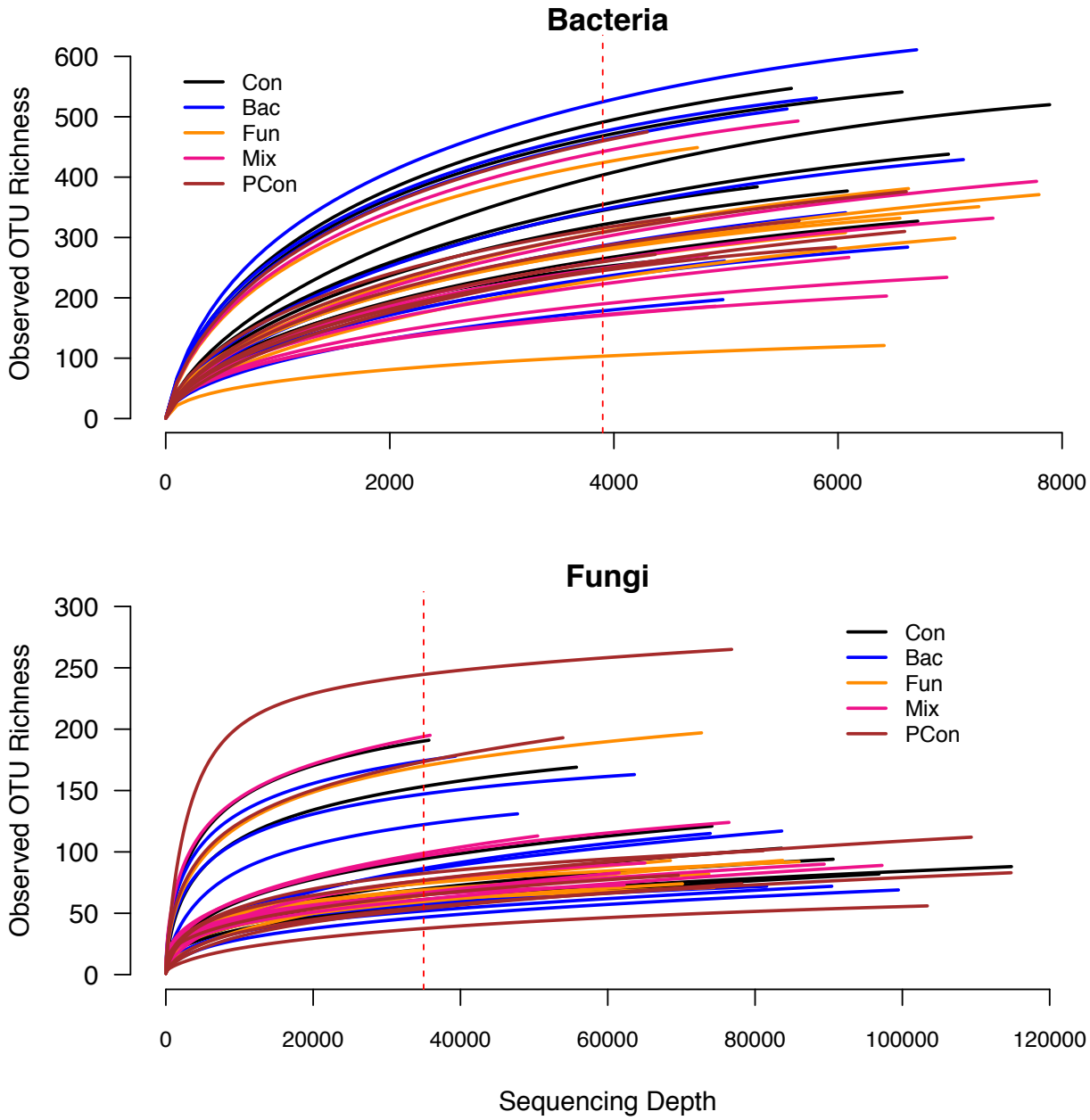

Figure S3: Rarefaction analysis of bacteria and fungi communities. Rarefaction curves were generated by random subsampling of sequencing counts from 0-8,000 (bacteria) and 0-115,000 sequences per sample in steps of 100 sequences. Lines are colored by experimental microbial treatment. The red dashed line indicates the rarefaction depth of 3,900 (bacteria) and 35,000 (fungi) sequences per sample applied to each respective dataset.

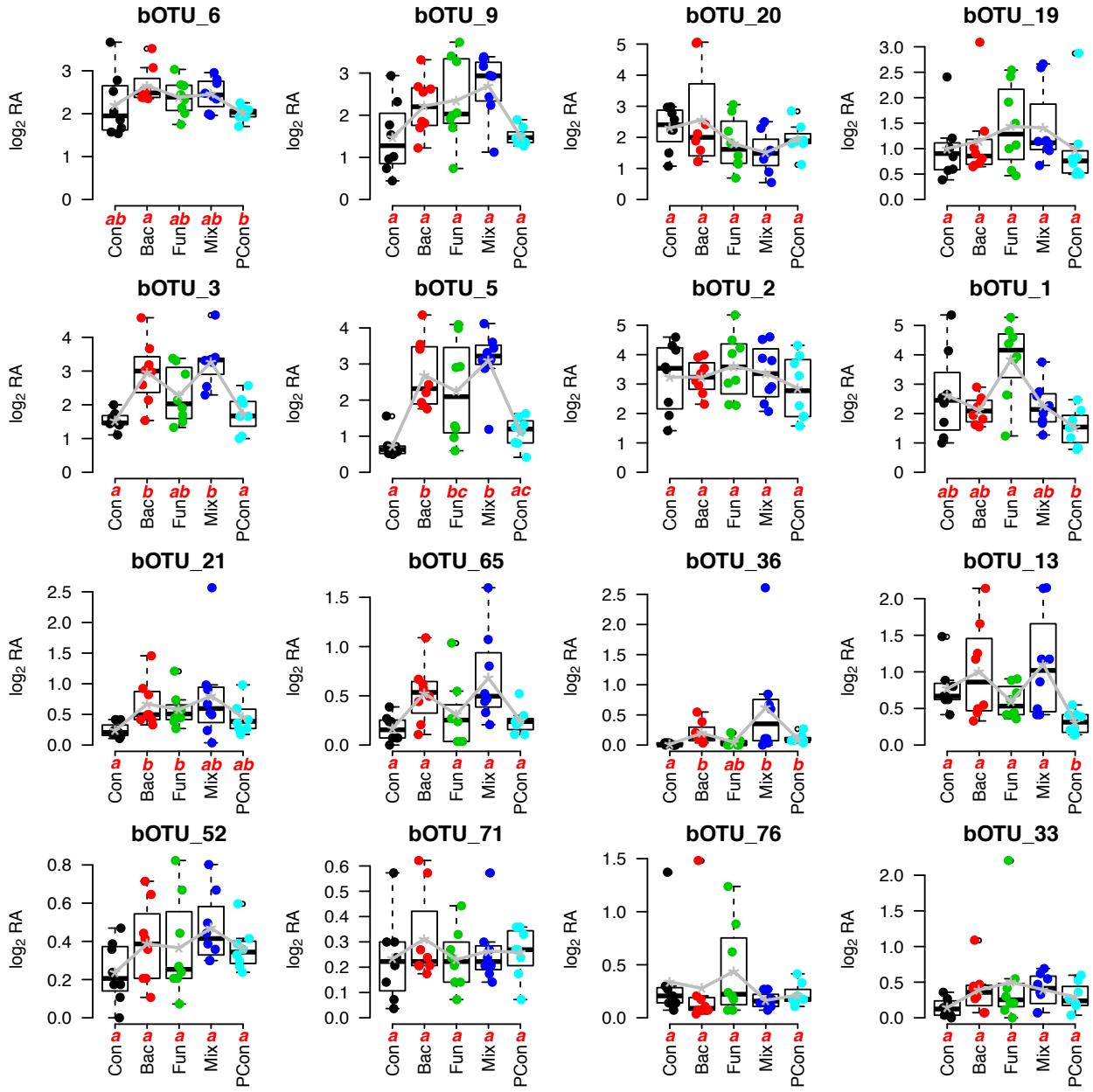

Figure S4: The CPM RAs of the inoculated bacteria isolate OTUs in the five microbial treatments. Gray stars indicate group means. Different letters indicate significant differences in RAs between the different groups as determined by a pairwise Wilcox test ($p<0.05$, FDR corrected).

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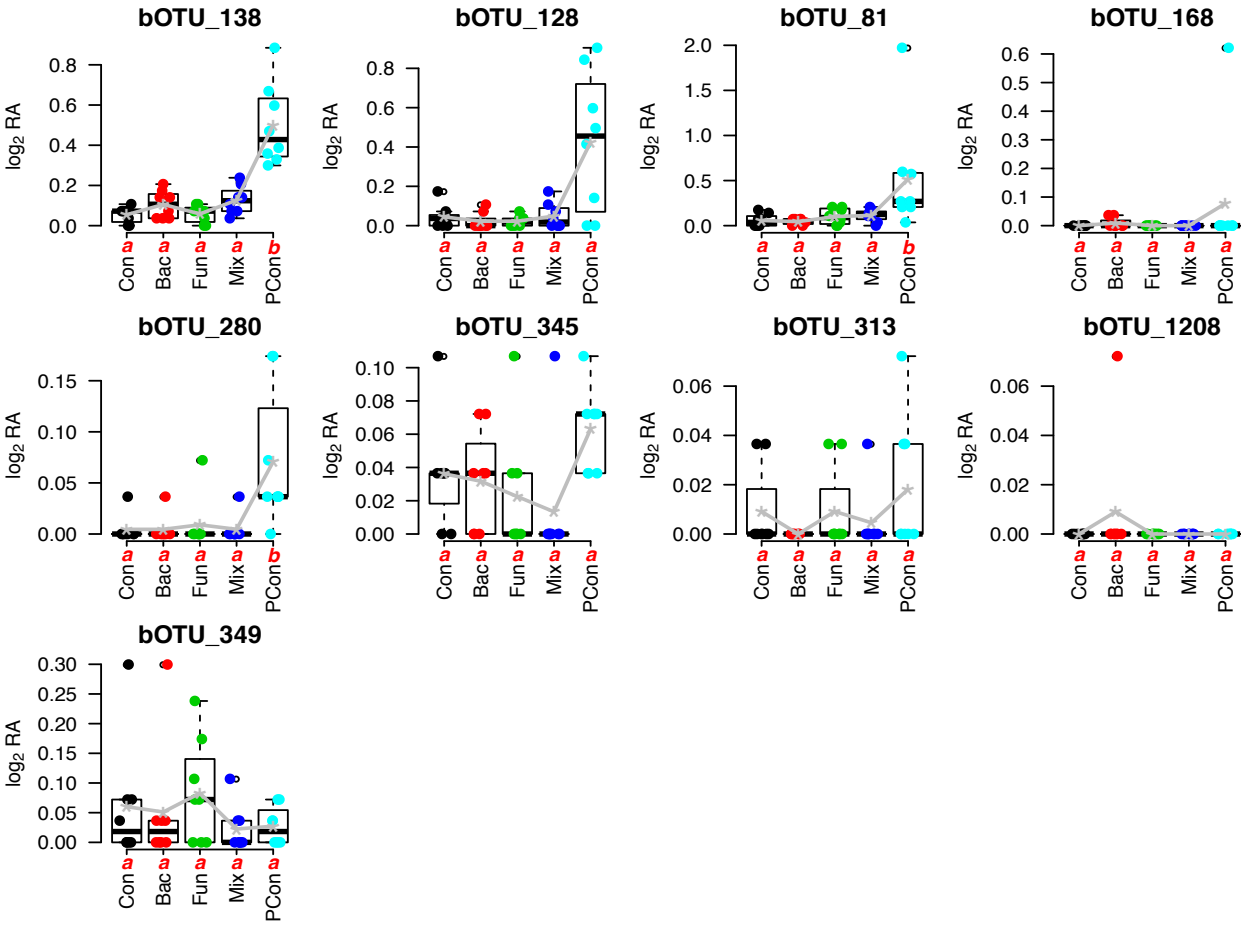

Figure S4 continued: The CPM RAs of the inoculated bacteria isolate OTUs in the five microbial treatments. Gray stars indicate group means. Different letters indicate significant differences in RAs between the different groups as determined by a pairwise Wilcox test ($p<0.05$, FDR corrected).

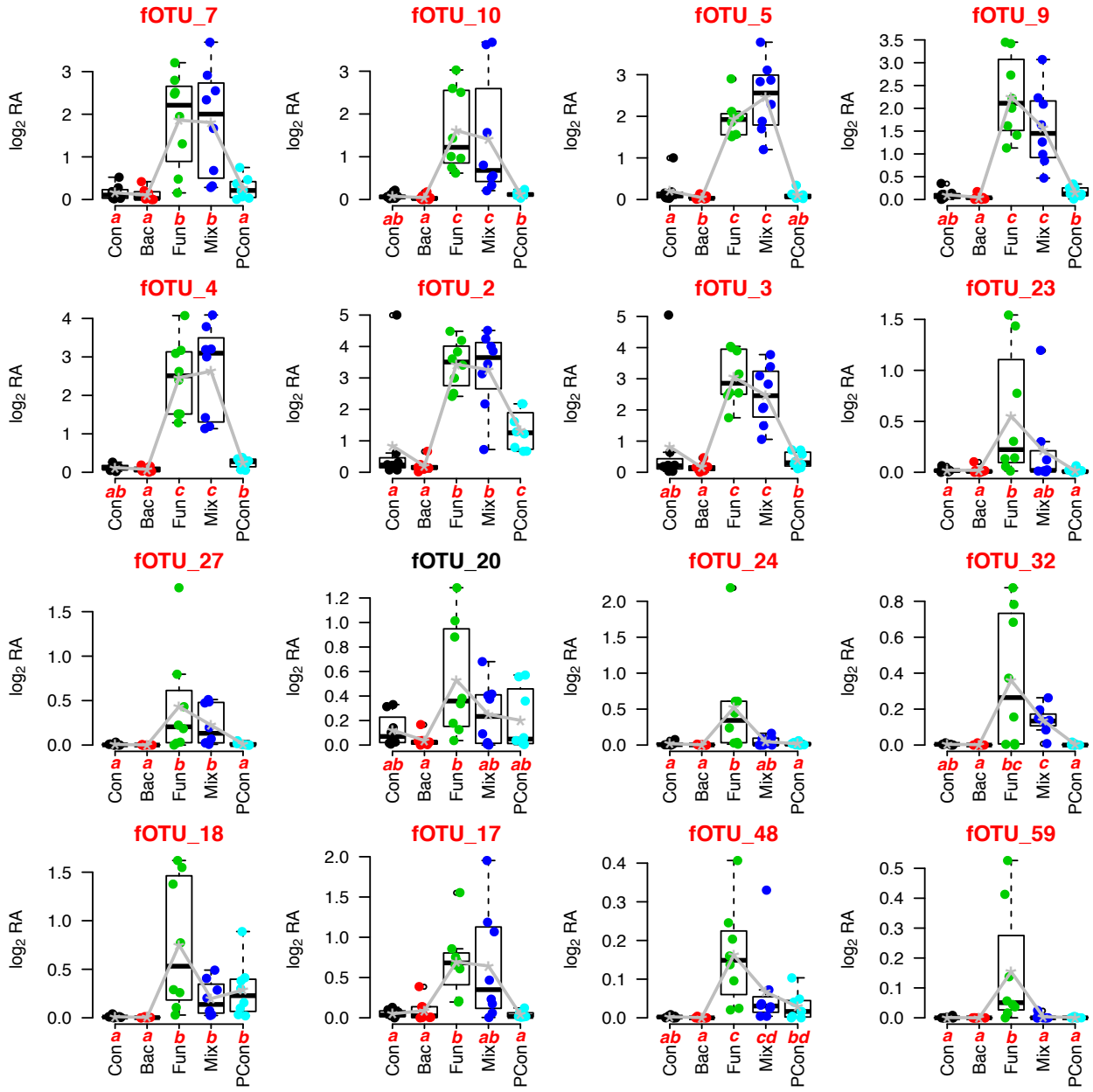

Figure S5: The CPM RAs of the inoculated fungi isolate OTUs in the five microbial treatments. Gray stars indicate group means. Different letters indicate significant differences in RAs between the different groups as determined by a pairwise Wilcox test ($p<0.05$, FDR corrected). OTU ID's in red indicate the inoculated fungi OTUs with significantly higher RAs in the *Fun* and/or *Mix* treatments compared to the non-inoculated *Con* and *Bac*.

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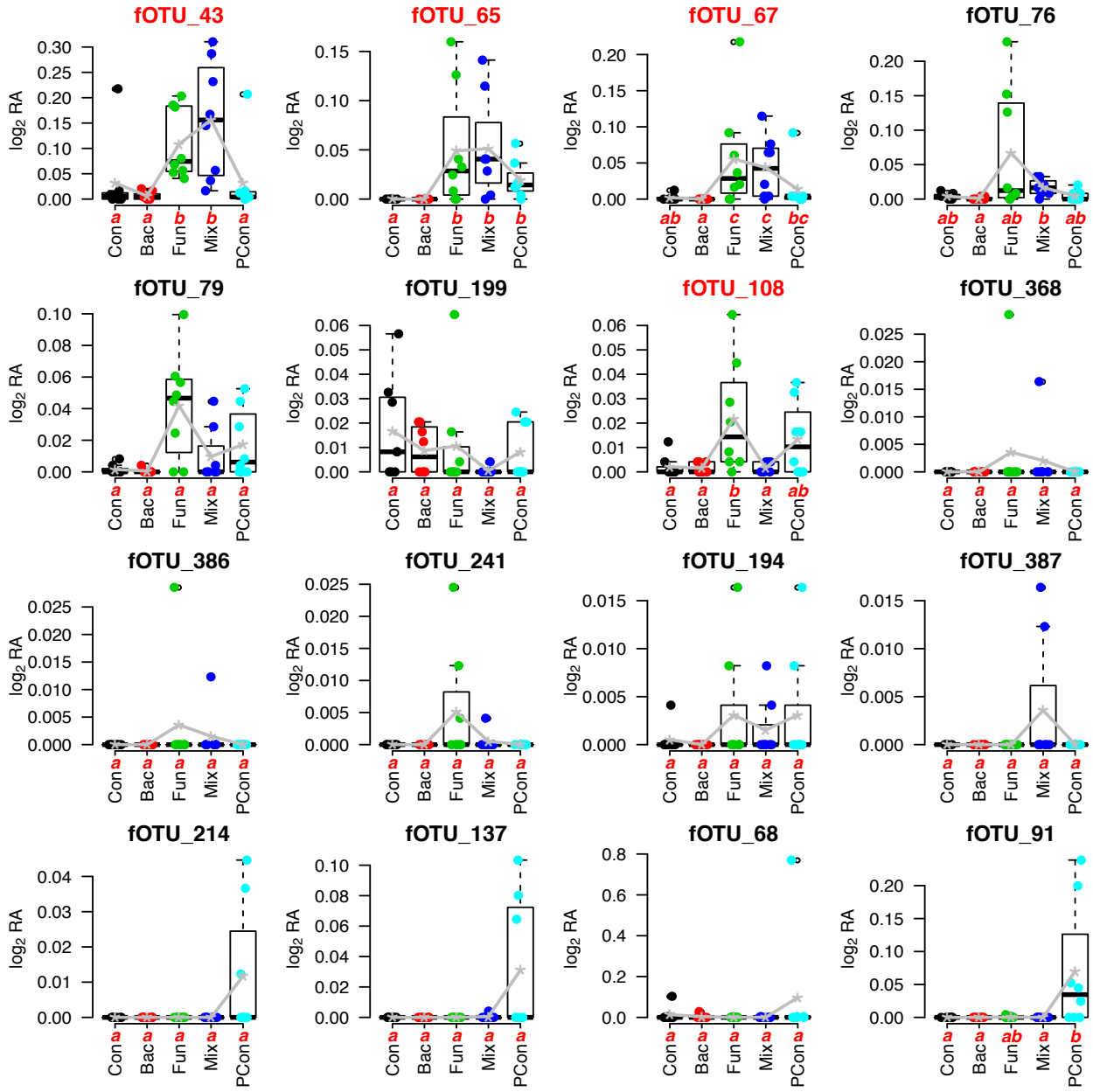

Fig S5 continued: The CPM RAs of the inoculated fungi isolate OTUs in the five microbial treatments. Gray stars indicate group means. Different letters indicate significant differences in RAs between the different groups as determined by a pairwise Wilcox test ($p<0.05$, FDR corrected). OTU ID's in red indicate the inoculated fungi OTUs with significantly higher RAs in the *Fun* and/or *Mix* treatments compared to the non-inoculated *Con* and *Bac*.

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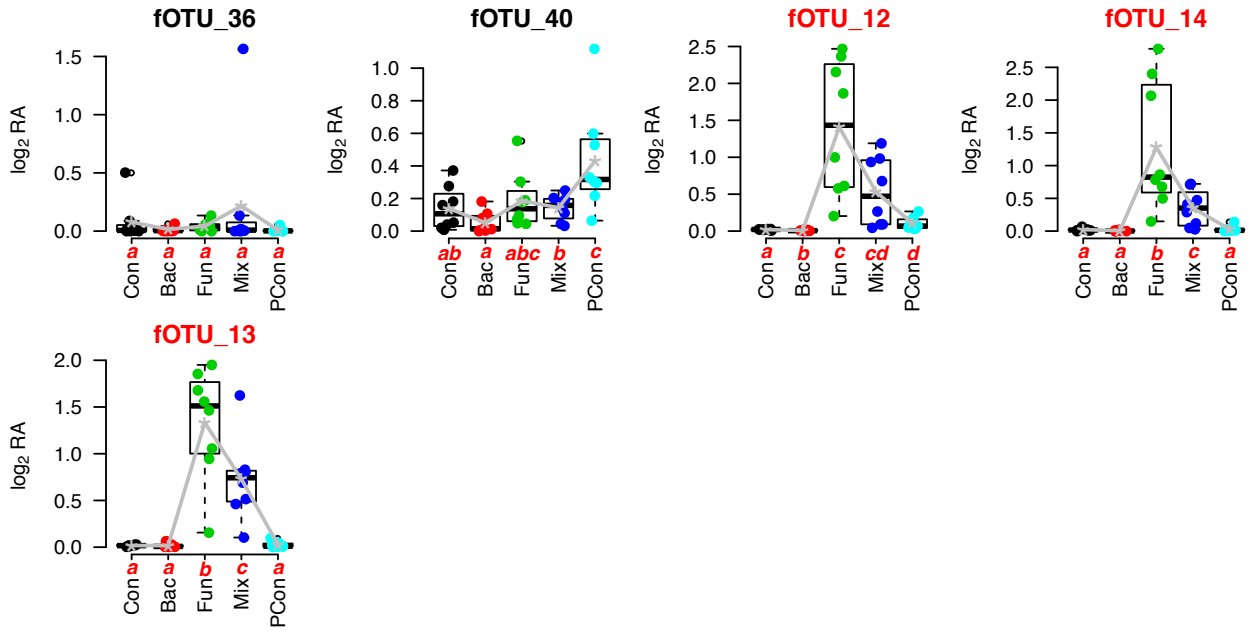

Figure S5 continued: The CPM RAs of the inoculated fungi isolate OTUs in the five microbial treatments. Gray stars indicate group means. Different letters indicate significant differences in RAs between the different groups as determined by a pairwise Wilcox test ($p<0.05$, FDR corrected). OTU ID's in red indicate the inoculated fungi OTUs with significantly higher RAs in the *Fun* and/or *Mix* treatments compared to the non-inoculated *Con* and *Bac*.

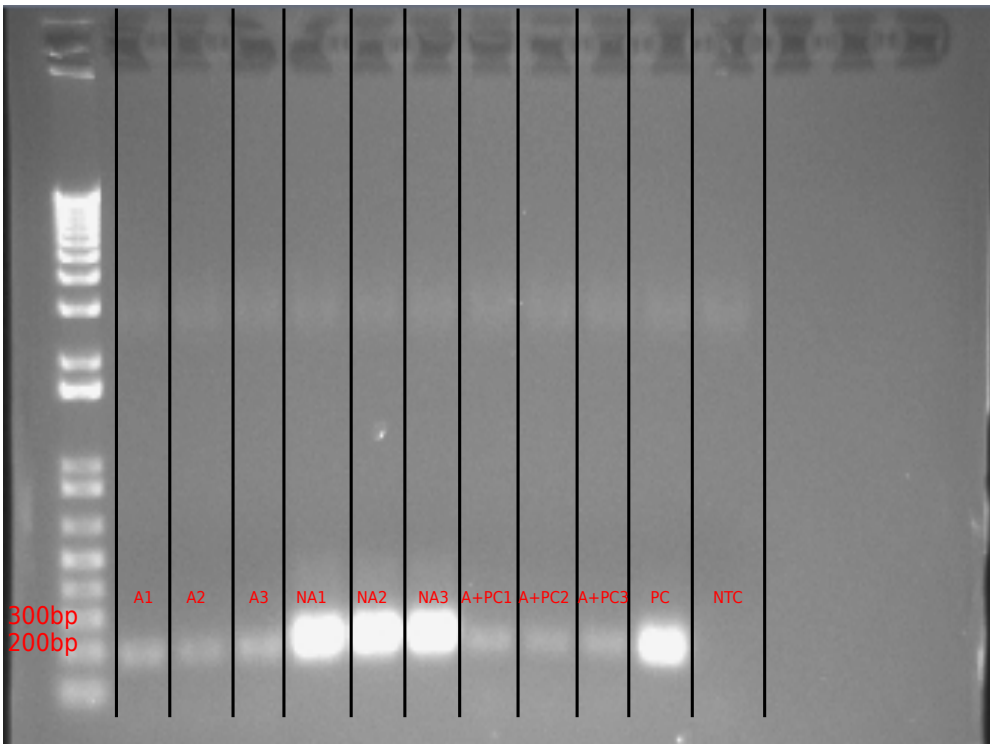

Figure S6: Agarose gel electrophoresis image of PCR testing for soil DNA viability before (**NA_{1-3}**) and after (**A_{1-3}**) autoclaving. **PC,** and **NTC** lanes show the positive control, and non-template control reactions, respectively. **A+PC** reactions contained DNA from autoclaved soil plus **PC** DNA to check for the presence of PCR inhibitors. Length of the generated amplicons is marked between 200 and 300bp.

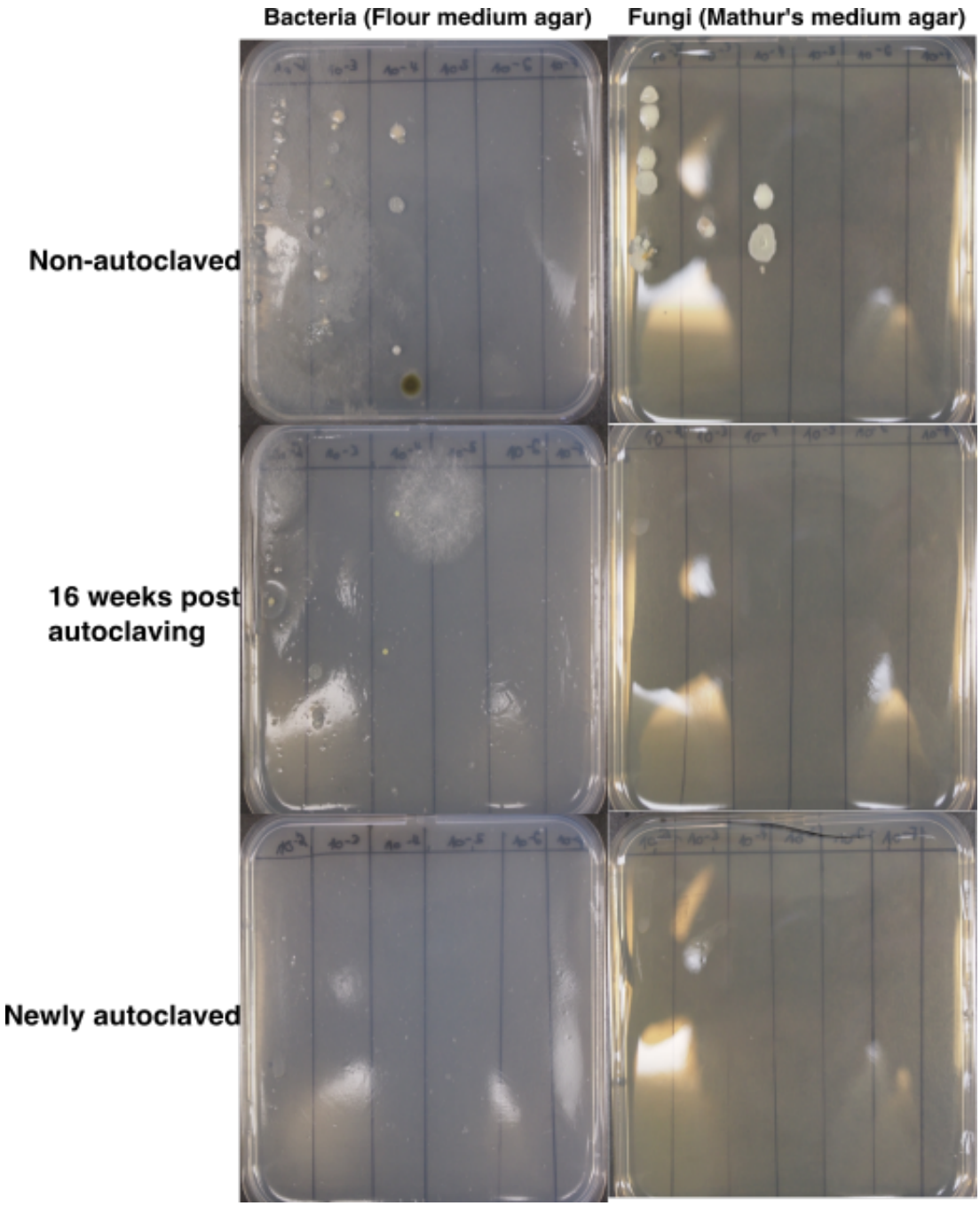

Figure S7: Agar plates of serial dilutions of non-autoclaved soil, autoclaved soil from microcosms after 16 weeks in the greenhouse, and newly autoclaved soil.

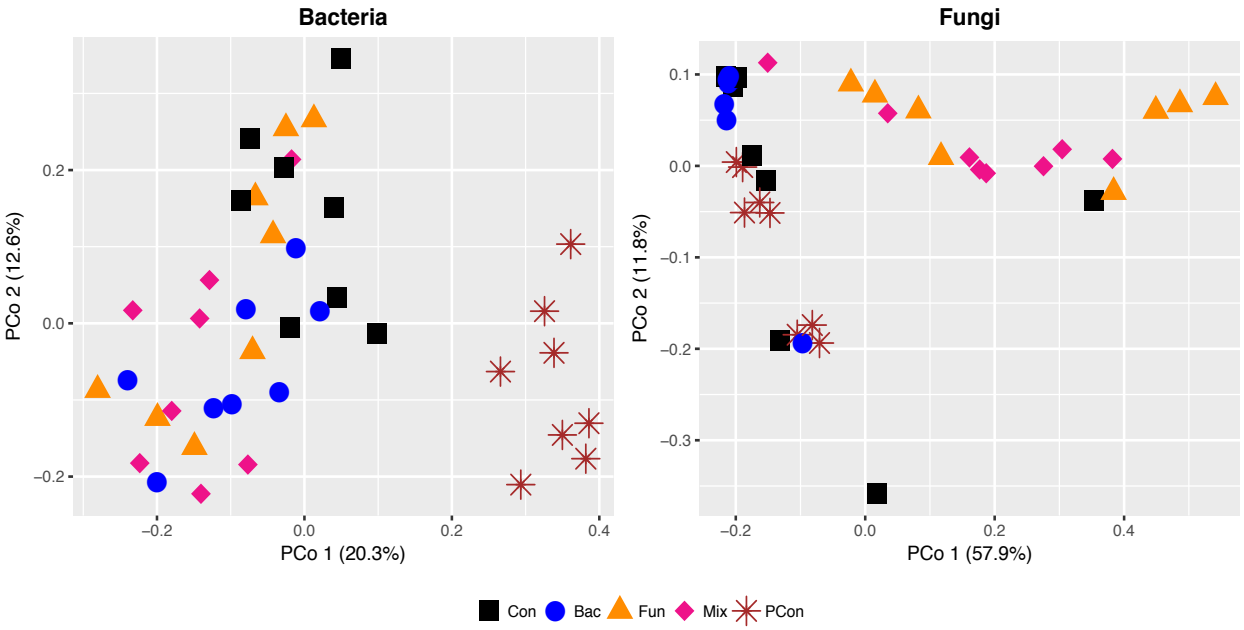

Figure S8: Unconstrained ordinations of the entire bacteria and fungi communities in the microcosms based on Bray-Curtis dissimilarities. Percentage of variation on each axis refers to the explained fraction of total variation in each indicated community.

Supplementary Tables

Table S1: PCR cycling conditions for identification of fungi isolates and 16S and ITS community profiling of microcosm soil samples.

Fungi Isolates				**Pre/post Autoclave DNA**				**16S Community Profiling**				**ITS Community Profiling**			
								preheated to 94ºC				preheated to 98ºC			
Step	Temp.	Time	Cycles	Step	Temp.	Time	Cycles	Step	Temp.	Time	Cycles	Step	Temp.	Time	Cycles
1	95°C	2min		1	95˚C	5min		1	94°C	3min		1	94°C	3min	
2	**94°C**	**1min**		**2**	**94˚C**	**1min**		**2**	**94°C**	**45sec**		**2**	**94°C**	**45sec**	
3	**54°C**	**1min**	**35x**	**3**	**53˚C**	**30sec**	**30x**	**3**	**55°C**	**30sec**	**30x**	**3**	**50ºC**	**1min**	**30x**
4	**72°C**	**1min**		**4**	**72˚C**	**1min**		**4**	**65°C**	**90sec**		**4**	**72°C**	**90sec**	
5	72°C	10min		5	72˚C	10min		5	65°C	10min		5	72°C	10min	
6	15°C	Hold		6	15˚C	Hold		6	15°C	Hold		6	15°C	Hold	

Table S2: Bacteria isolates clustering to a bacteria OTU in the community profiles. NAs indicate no community OTU was found for that isolate. OTU mean relative abundance (%) ± SEM are given for each microbial treatment. Relative abundance values for each microcosm and the results of statistical testing are shown in Fig. S4.

Isolate	OTU	SeqSim	Con	Bac	Fun	Mix	PCon	Phylum	Class	Order	Family	Genus	Species
733A3	bOTU_2	100	11.1 ± 2.82	9.08 ± 1.39	14.66 ± 4.28	11.46 ± 2.79	8.19 ± 2.27	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	unassigned
927D1		99.7											
558B3		99.2											
933F1		98.9											
217B	bOTU_1208	97.5	0 ± 0	0.01 ± 0.01	0 ± 0	0 ± 0	0 ± 0	Planctomycetes	vadinHA49	unassigned	unassigned	unassigned	unassigned
714B3		97.2											
856C1	bOTU_20	98.9	4.36 ± 0.77	9.88 ± 4.84	3.04 ± 0.87	2.12 ± 0.51	3.06 ± 0.52	Proteobacteria	α-proteobacteria	unassigned	unassigned	unassigned	unassigned
199B4		98.1											
737E3	bOTU_21	100	0.18 ± 0.03	0.64 ± 0.18	0.52 ± 0.12	1.05 ± 0.56	0.39 ± 0.1	Proteobacteria	β-proteobacteria	unassigned	unassigned	unassigned	unassigned
601C3		97.2											
722B4	bOTU_280	100	0 ± 0	0 ± 0	0.01 ± 0.01	0 ± 0	0.05 ± 0.02	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	unassigned	unassigned
737A3		97.8											
218F	bOTU_33	100	0.11 ± 0.03	0.34 ± 0.12	0.63 ± 0.43	0.33 ± 0.07	0.23 ± 0.06	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
718D3		99.2											
276C	bOTU_1	100	9.42 ± 4.71	3.56 ± 0.55	17.75 ± 4.19	4.51 ± 1.25	2.11 ± 0.46	Proteobacteria	δ-proteobacteria	Myxococcales	unassigned	unassigned	unassigned
954A1	bOTU_128	99.4	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.38 ± 0.12	Proteobacteria	δ-proteobacteria	Myxococcales	0319-6G20	unassigned	unassigned
203A3	bOTU_13	99.4	0.75 ± 0.16	1.21 ± 0.39	0.53 ± 0.08	1.41 ± 0.46	0.25 ± 0.05	Proteobacteria	α-proteobacteria	Rhodospirillales	I-10	unassigned	unassigned
606A3	bOTU_138	100	0.04 ± 0.01	0.08 ± 0.02	0.04 ± 0.01	0.09 ± 0.02	0.43 ± 0.07	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Terrabacter	unassigned
362B3	bOTU_168	100	0 ± 0	0.01 ± 0	0 ± 0	0 ± 0	0.07 ± 0.07	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	env.OPS 17	unassigned	unassigned
174B4	bOTU_19	100	1.21 ± 0.46	1.69 ± 0.84	2.09 ± 0.61	2.06 ± 0.69	1.36 ± 0.72	Proteobacteria	α-proteobacteria	Rhizobiales	Xanthobacteraceae	unassigned	unassigned
202B	bOTU_3	100	1.93 ± 0.19	8.38 ± 2.33	4.57 ± 1.14	9.81 ± 2.21	2.52 ± 0.46	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter	fermentans
616A3	bOTU_313	99.4	0.01 ± 0	0 ± 0	0.01 ± 0	0 ± 0	0.01 ± 0.01	Bacteroidetes	unassigned	unassigned	unassigned	unassigned	unassigned

Table continues on next page

929A1	bOTU_345	98.3	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.04 ± 0.01	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
941C1	bOTU_349	97.5	0.04 ± 0.03	0.04 ± 0.03	0.06 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	Proteobacteria	Deltaproteobacteria	GR-WP33-30	unassigned	unassigned	unassigned
722C3	bOTU_36	98.3	0.01 ± 0	0.15 ± 0.05	0.04 ± 0.02	0.9 ± 0.61	0.08 ± 0.02	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
274A	bOTU_5	99.4	0.71 ± 0.18	6.96 ± 2.13	6.03 ± 2.23	8.66 ± 1.52	1.24 ± 0.21	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	env.OPS 17	unassigned	unassigned
605A3	bOTU_52	100	0.19 ± 0.05	0.32 ± 0.07	0.31 ± 0.09	0.39 ± 0.06	0.29 ± 0.04	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	Acidiferrobacter	unassigned
198B	bOTU_6	99.7	4.26 ± 1.17	5.57 ± 0.8	4.39 ± 0.55	4.63 ± 0.49	3.04 ± 0.16	Planctomycetes	Pla4 lineage	unassigned	unassigned	unassigned	unassigned
856A1	bOTU_65	99.7	0.13 ± 0.04	0.47 ± 0.11	0.27 ± 0.12	0.68 ± 0.22	0.19 ± 0.04	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	env.OPS 17	unassigned	unassigned
607A3	bOTU_71	99.4	0.18 ± 0.05	0.25 ± 0.06	0.18 ± 0.03	0.21 ± 0.04	0.2 ± 0.03	Actinobacteria	MB-A2-108	unassigned	unassigned	unassigned	unassigned
737D3	bOTU_76	100	0.32 ± 0.18	0.29 ± 0.22	0.41 ± 0.17	0.12 ± 0.02	0.16 ± 0.03	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Bdellovibrio	unassigned
219B3	bOTU_81	100	0.04 ± 0.02	0.03 ± 0.01	0.07 ± 0.02	0.08 ± 0.02	0.58 ± 0.34	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	unassigned	unassigned
741A3	bOTU_9	100	2.25 ± 0.76	4.13 ± 0.87	5.29 ± 1.5	6.15 ± 1.01	1.86 ± 0.15	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Incertae Sedis	unassigned
202A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
366A3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
633B3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
607B3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
359A3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
271F	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
287A3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
736B3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
742A3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
941E3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
293A3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
954B1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
281A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
359B3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
286A3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Table S3: Fungi isolates clustering to a fungi OTU in the community profiles and their sequence similarity. NAs indicate no community OTU was found for that isolate. OTU mean relative abundance (%) ± SEM are given for each microbial treatment. OTU names in **bold** were determined to have a significantly higher RA in *Fun* and/or *Mix* treatments compared to the *Con* and *Bac* (see Fig. S5).

Isolate	OTU	SeqSim (%)	Con	Bac	Fun	Mix	PCon	Phylum	Class	Order	Family	Genus	Species
477B	**fOTU_10**	99.3	0.06 ± 0.02	0.03 ± 0.02	2.72 ± 0.9	3.39 ± 1.79	0.09 ± 0.02	Ascomycota	Sordariomycetes	Hypocreales	Incertae sedis	Acremonium	persicinum
505A_3													
188C_4	**fOTU_2**	100	4.06 ± 3.86	0.16 ± 0.06	11.09 ± 2.13	11.27 ± 2.54	1.72 ± 0.43	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
529B_3		98.6											
580B_3	**fOTU_108**	99.3	0 ± 0	0 ± 0	0.02 ± 0.01	0 ± 0	0.01 ± 0	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
833A_3	**fOTU_12**	97.9	0.01 ± 0	0.01 ± 0	2.12 ± 0.63	0.52 ± 0.17	0.08 ± 0.02	Ascomycota	unassigned	unassigned	unassigned	unassigned	unassigned
834B_3	**fOTU_13**	97.9	0.01 ± 0	0.01 ± 0.01	1.69 ± 0.33	0.73 ± 0.21	0.02 ± 0.01	Ascomycota	Sordariomycetes	Sordariales	Cephalothecaceae	Phialemonium	atrogriseum
641A_3	fOTU_137	98.6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.01	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
834C_3	**fOTU_14**	100	0.02 ± 0.01	0 ± 0	2 ± 0.77	0.3 ± 0.09	0.02 ± 0.01	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	abidjanum
127A_4	**fOTU_17**	100	0.04 ± 0.01	0.07 ± 0.04	0.68 ± 0.2	0.75 ± 0.35	0.03 ± 0.01	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	unassigned	unassigned
318A_3	**fOTU_18**	99.3	0.01 ± 0	0 ± 0	0.85 ± 0.31	0.16 ± 0.05	0.24 ± 0.1	Ascomycota	unassigned	unassigned	unassigned	unassigned	unassigned
647C	fOTU_194	99.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Ascomycota	Leotiomycetes	Erysiphales	Erysiphaceae	Erysiphe	unassigned
126A_4	fOTU_199	97.8	0.01 ± 0.01	0.01 ± 0	0.01 ± 0.01	0 ± 0	0.01 ± 0	Ascomycota	unassigned	unassigned	unassigned	unassigned	unassigned
650A	fOTU_20	100	0.09 ± 0.04	0.03 ± 0.01	0.51 ± 0.18	0.21 ± 0.08	0.17 ± 0.08	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
694C_3	fOTU_214	99.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.01 ± 0	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
160B_4	**fOTU_23**	100	0.01 ± 0.01	0.01 ± 0.01	0.6 ± 0.28	0.21 ± 0.16	0.01 ± 0.01	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
184B_4	**fOTU_24**	100	0.01 ± 0.01	0 ± 0	0.65 ± 0.42	0.03 ± 0.02	0.01 ± 0.01	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
420A	fOTU_241	99.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	unassigned	unassigned
552C	**fOTU_27**	100	0 ± 0	0 ± 0	0.48 ± 0.29	0.18 ± 0.07	0.01 ± 0	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
188B_4	**fOTU_3**	99.3	4.16 ± 3.98	0.12 ± 0.04	8.61 ± 1.89	5.63 ± 1.44	0.33 ± 0.08	Ascomycota	unassigned	unassigned	unassigned	unassigned	unassigned
126C_4	**fOTU_32**	100	0 ± 0	0 ± 0	0.32 ± 0.12	0.1 ± 0.02	0 ± 0	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	unassigned	unassigned

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233A_4	fOTU_36	98.6	0.06 ± 0.05	0.01 ± 0.01	0.03 ± 0.01	0.26 ± 0.24	0.01 ± 0	Ascomycota	unassigned	unassigned	unassigned	unassigned	unassigned
563A	fOTU_368	98.6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
582B	fOTU_386	100	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
423A_3	fOTU_387	100	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
842A_3	**fOTU_4**	99.3	0.09 ± 0.02	0.06 ± 0.02	5.75 ± 1.7	7.05 ± 1.95	0.19 ± 0.04	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
836D_3	fOTU_40	100	0.11 ± 0.04	0.04 ± 0.02	0.15 ± 0.05	0.11 ± 0.02	0.38 ± 0.12	Ascomycota	Sordariomycetes	Chaetosphaeriales	Chaetosphaeriaceae	unassigned	unassigned
646B	**fOTU_43**	99.3	0.02 ± 0.02	0 ± 0	0.08 ± 0.02	0.12 ± 0.03	0.02 ± 0.02	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
547A	**fOTU_48**	99.3	0 ± 0	0 ± 0	0.12 ± 0.04	0.05 ± 0.03	0.02 ± 0.01	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
306C_3	**fOTU_5**	100	0.18 ± 0.12	0.03 ± 0.01	3.02 ± 0.53	5.37 ± 1.31	0.08 ± 0.03	Ascomycota	Sordariomycetes	Glomerellales	Glomerellaceae	Colletotrichum	unassigned
161D_4	**fOTU_59**	100	0 ± 0	0 ± 0	0.12 ± 0.06	0 ± 0	0 ± 0	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
333A_3	**fOTU_65**	100	0 ± 0	0 ± 0	0.04 ± 0.02	0.04 ± 0.01	0.01 ± 0	Ascomycota	Dothideomycetes	Capnodiales	Incertae sedis	unidentified	unassigned
418A	**fOTU_67**	100	0 ± 0	0 ± 0	0.04 ± 0.02	0.03 ± 0.01	0.01 ± 0.01	Ascomycota	unassigned	unassigned	unassigned	unassigned	unassigned
113A_4	fOTU_68	100	0.01 ± 0.01	0 ± 0	0 ± 0	0 ± 0	0.09 ± 0.09	unassigned	unassigned	unassigned	unassigned	unassigned	unassigned
118A_4	**fOTU_7**	100	0.12 ± 0.05	0.08 ± 0.04	3.54 ± 1.01	3.82 ± 1.42	0.22 ± 0.08	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinopsis	semitalis
834A_3	fOTU_76	100	0 ± 0	0 ± 0	0.05 ± 0.02	0.01 ± 0	0 ± 0	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
659B	fOTU_79	98.6	0 ± 0	0 ± 0	0.03 ± 0.01	0.01 ± 0	0.01 ± 0.01	Ascomycota	unassigned	unassigned	unassigned	unassigned	unassigned
232B_4	**fOTU_9**	100	0.08 ± 0.03	0.03 ± 0.01	4.6 ± 1.24	2.5 ± 0.81	0.12 ± 0.03	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	unassigned
563C	fOTU_91	99.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.05 ± 0.03	Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Neobulgaria	unassigned
469A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
520A_3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
430A_3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
238B_4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
499C	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
400A_3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Table S4: ANOVA and Tukey pairwise comparisons of inoculated bacteria and fungi OTU richness and total relative abundance (RA) across the five microbial treatments. Significant effects are indicated in bold (*$p<0.05$, **$p>0.01$, ***$p<0.001$). Different letters in the Tukey pairwise comparisons indicate significant differences at $p<0.05$. Data are plotted in Figs. 3 and 4 in the main text.

	Inoculated OTU Richness		**Inoculated OTUs RA(%)**	
	Bacteria	**Fungi**	**Bacteria**	**Fungi**
Microbial Treatment $_{(4,32)}$	***F=5.44*** **	***F=32.15*** ***	***F=11.07*** ***	***F=15.29*** ***
Pairwise Comparisons	**Mean ± SEM**		**Mean ± SEM**	
Con	18.5 ± 0.63 **(a)**	20.88 ± 0.64 **(a)**	37.29 ± 5.09 **(a)**	9.18 ± 7.86 **(a)**
Bac	19.63 ± 0.42 **(ab)**	16.25 ± 1.03 **(b)**	53.12 ± 4.93 **(ab)**	0.71 ± 0.2 **(a)**
Fun	18.75 ± 0.73 **(a)**	26.75 ± 0.82 **(c)**	60.93 ± 3.25 **(b)**	49.93 ± 7.7 **(b)**
Mix	19 ± 0.53 **(a)**	24.88 0.64 **(c)**	54.73 ± 4.57 **(b)**	42.84 ± 6.15 **(b)**
PCon	21.63 ± 0.46 **(b)**	24 ± 0.68 **(c)**	26.74 ± 2.15 **(ac)**	3.99 ± 0.66 **(a)**

Table S5: ANOVA and Tukey pairwise comparisons of total bacteria and fungi OTU richness across the five microbial treatments. Significant effects are indicated in bold (*$p<0.05$, **$p>0.01$, ***$p<0.001$). Different letters in the Tukey pairwise comparisons indicate significant differences at $p<0.05$.

	Bacteria	**Fungi**
Microbial Treatment $_{(4,32)}$	***F=23.23****	***F=15.07****
Pairwise Comparisons	**Mean ± SEM**	
Con	255.25 ± 19.48 **(a)**	94.88 ± 23.34 **(a)**
Bac	266 ± 17.24 **(a)**	68 ± 5.9 **(a)**
Fun	264.88 ± 27.97 **(a)**	69.38 ± 3 **(a)**
Mix	269 ± 23.97 **(a)**	68.5 ± 3.98 **(a)**
PCon	470.63 ± 10.6 **(b)**	165.63 ± 7.88 **(b)**

Table S6: Results of PERMANOVA testing the effects of *Block* and *Microbial Treatment* on bacteria and fungi communities in microcosm soil samples. Significant effects are indicated in bold (*$p<0.05$, **$p>0.01$, ***$p<0.001$). Results of BETADISP testing for differences in multivariate dispersion between *Block* and *Microbial Treatment* in bacteria and fungi communities. Different letters in the pairwise comparisons indicate significant differences at $p<0.05$ (FDR corrected).

PERMANOVA

	Bacteria		**Fungi**	
Factor	**pseudo-F**	**R^2**	**pseudo-F**	**R^2**
Block $_{(3,32)}$	***1.54****	***0.09***	0.93	0.04
Microbial Treatment $_{(4,32)}$	***4.37*****	***0.32***	***7.86*****	***0.47***
Pairwise Block				
	Block 1 ***(a)***		-	
	Block 2 ***(a)***		-	
	Block 3 ***(a)***		-	
	Block 4 ***(a)***		-	
Pairwise Microbial Treatment				
	Con ***(a)***		*Con* ***(a)***	
	Bac ***(b)***		*Bac* ***(a)***	
	Fun ***(c)***		*Fun* ***(b)***	
	Mix ***(b)***		*Mix* ***(b)***	
	PCon ***(d)***		*PCon* ***(c)***	
Multivariate homogeneity of groups dispersions				
Block $_{(3,36)}$	1.34		-	
Microbial Treatment $_{(4,35)}$	***8.57*****		***6.07****	
Pairwise Dispersion				
	Con ***(a)***		*Con* ***(a)***	
	Bac ***(ab)***		*Bac* ***(b)***	
	Fun ***(ab)***		*Fun* ***(a)***	
	Mix ***(b)***		*Mix* ***(a)***	
	PCon ***(c)***		*PCon* ***(b)***	

Table S7: ANOVA table of effects of *Plant Mortality* and *Microbial Treatment* on the measured ecosystem functions. Significant effects are indicated in bold (*$p<0.05$, **$p>0.01$, ***$p<0.001$). Different letters in the Tukey pairwise comparisons indicate significant differences at $p<0.05$.

	Primary Productivity		**Decomposition**		**Leaching Volume**		**N-N_2O loss**		**Multi-functionality**	
	df	**F**	**df**	**F**	**df**	**F**	**df**	**F**	**df**	**F**
Plant Mortality	1,34	2.55	-	-	-	-	-	-	-	-
Microbial Treatment	4,31	***2.64****	4,32	***7.96*****	4,32	1.82	4,32	0.98	4,32	***3.33****

Pairwise Comparisons	**Mean ± SEM**				
	g biomass microcosm^{-1}	**Litter lost %**	**H_2O lost %**	**µg N lost microcosm^{-1}**	**Multi-functionality Index**
Con	26.34 ± 0.72 ***(a)***	54.7 ± 4.26 ***(a)***	39.77 ± 3.16 ***(a)***	1.17 ± 0.49 ***(a)***	0.32 ± 0.09 ***(ac)***
Bac	23.99 ± 0.86 ***(ab)***	55.75 ± 5.91 ***(a)***	50.57 ± 2.81 ***(a)***	4.11 ± 2.57 ***(a)***	-0.28 ± 0.22 ***(abc)***
Fun	23.46 ± 0.81 ***(ab)***	62.7 ± 2.29 ***(a)***	45.43 ± 3.95 ***(a)***	1.73 ± 0.76 ***(a)***	0.02 ± 0.16 ***(abc)***
Mix	22.68 ± 0.96 ***(b)***	60.28 2.93 ***(a)***	53.20 ± 5.36 ***(a)***	5.07 ± 3.11 ***(a)***	-0.44 ± 0-31 ***(b)***
PCon	23.91 ± 0.59 ***(ab)***	81.37 ± 0.97 ***(b)***	48.08 ± 3.39 ***(a)***	0.98 ± 0.28 ***(a)***	0.38 ± 0.1 ***(c)***

Table S8: Spearman's rho (ρ) and p-values (FDR corrected) of correlations between RAs of the 22 fungi isolate-OTUs with enriched abundance in *Fun* and/or *Mix* treatments (Fig. S5, Table S3) and the measured ecosystem functioning responses.

	Biomass		**Decomposition**		**Leaching**		**N-N_2O Loss**	
	ρ	**P-value**	**ρ**	**P-value**	**ρ**	**P-value**	**ρ**	**P-value**
fOTU_7	-0.2	0.46	0.06	0.96	0.15	0.97	0.19	0.8
fOTU_10	-0.28	0.32	0.03	0.96	0.02	0.97	0.18	0.8
fOTU_2	-0.31	0.32	0.32	0.47	0.02	0.97	-0.06	0.89
fOTU_48	0.23	0.44	-0.13	0.92	-0.19	0.97	0.11	0.85
fOTU_27	0.22	0.44	-0.01	0.96	0.17	0.97	-0.14	0.8
fOTU_23	0.16	0.51	0.12	0.92	0.01	0.97	0.2	0.8
fOTU_65	0.27	0.32	-0.36	0.47	0.06	0.97	0.11	0.85
fOTU_18	-0.19	0.46	0.01	0.96	0.08	0.97	-0.16	0.8
fOTU_43	-0.01	0.95	0.24	0.79	-0.19	0.97	-0.23	0.8
fOTU_12	-0.15	0.51	0.2	0.91	0.03	0.97	-0.15	0.8
fOTU_13	-0.16	0.51	0.07	0.96	-0.12	0.97	0.04	0.89
fOTU_14	0.15	0.51	-0.06	0.96	-0.07	0.97	0.04	0.89
fOTU_4	-0.36	0.32	0.14	0.92	0.06	0.97	0.04	0.89
fOTU_32	0.01	0.95	-0.07	0.96	0.19	0.97	0.31	0.57
fOTU_108	0.32	0.32	-0.15	0.92	0.19	0.97	0.01	0.97
fOTU_3	-0.18	0.5	0.15	0.92	0	0.99	-0.06	0.89
fOTU_24	0.11	0.66	0.03	0.96	-0.02	0.97	0.34	0.57
fOTU_59	0.3	0.32	-0.01	0.96	0.04	0.97	-0.15	0.8
fOTU_5	-0.08	0.75	-0.01	0.96	-0.03	0.97	0.07	0.89
fOTU_9	-0.26	0.35	0.18	0.92	-0.03	0.97	0.03	0.89
fOTU_17	0.02	0.95	-0.06	0.96	-0.18	0.97	-0.09	0.88
fOTU_67	0.06	0.83	-0.24	0.79	-0.08	0.97	0.25	0.8

Chapter 5:
General Discussion

The modern, intensive agricultural practices developed during the "Green Revolution" of the 1960s have increased the productivity of staple crops to meet the demands of a population that has simultaneously doubled in size [1]. However, it has been estimated that agricultural production will have to increase between 25 and 70% by 2050 to meet global demands [2,3]. Previous gains in agricultural production have primarily been attributed to increased application of phosphorus (P) and nitrogen (N) fertilizers [4]. However, recent estimates have suggested that nonrenewable phosphate rock stocks could be exhausted in the next 50-100 years [5] with estimates of peak production as early as 2033 [6]. Additionally, production of N fertilizer consumes approximately 1% of the world's annual energy supply and is a substantial contributor to worldwide greenhouse gas (GHG) emissions [7]. A growing body of evidence has suggested that modern conventional agriculture contributes to increased GHG emissions and nutrient leaching as a result of intensive fertilizer application [8], increased soil erosion [9], and detrimental effects on biodiversity [10,11]. Thus, an increase in agricultural output will have to be met with a reduction in the impact of agricultural practices on the natural environment. Addressing these challenges requires an ecological intensification of agriculture which aims to manage organisms that make a quantifiable direct or indirect contribution to agricultural production [12]. Given the vast microbial diversity of soils and the role that microorganisms play in a number of ecosystem functions, harnessing power of microorganisms has been suggested as a way to increase the productivity and sustainability of agricultural land [13]. However, there are many unanswered questions about how agricultural practices influence soil and root microbiomes. Additionally, given that the functions of many microbes are still unknown, harnessing the power of the microbiome requires methods for linking its composition to its function. Achieving the maximum benefit from the microbiome requires the application of management strategies at different scales, which include determining which agricultural practices promote conditions for beneficial microbes and support overall soil biodiversity but also focusing on targeted manipulations with the aim of altering specific ecosystem processes that may be beneficial for plant or overall agroecosystem functioning [14]. Meeting such a goal requires investigating how agriculture affects soil and root microbiomes and gaining insights into the functions of microbiome members both individually and in a community context.

With this in mind, this thesis aimed to investigate the effects of conventional and organic agriculture with varying tillage intensities on both soil and root microbial communities. Combined with reference stocks of bacteria and fungi isolates, I have performed experiments employing reductionist and holistic approaches that begin to investigate the function and importance of both root and soil microbial communities for plant and overall agroecosystem functioning. Together, these approaches contribute to a new generation of research that could be used to improve the sustainability of agricultural production.

Soil and root microbial communities respond differently to agricultural management

In Chapter 2, we used high throughput DNA sequencing to investigate how conventional and organic agricultural practices, combined with different tillage intensities, affect the richness and composition of soil and root bacteria and fungi communities in winter wheat. The effects of agricultural management on microbial communities have typically been focused on examining single factors like production system (conventional vs. organic) type [15–17], tillage intensities [18–20], or soil amendments [21–23] on either the soil bacteria or fungi community. However, the work presented here is important because it not only examines the effects of how the four different cropping systems - combining different production systems and tillage treatments - of the FAST experiment influence richness and composition of soil communities but also *root* microbial communities. We observed that the effects of the different cropping systems on microbial communities were greater for community composition than richness and depended on the kingdom and compartment being examined. While differences in tillage were the driving factor behind soil bacteria community composition, differences in production system drove differences in the root bacteria community. The opposite was observed for the fungi community, with production system driving the soil fungi community and tillage being the most important factor in the root fungi community.

The composition of the root microbiome has a direct influence on the health and productivity of the host-plant [24]. It is therefore surprising that only a few studies have specifically investigated how agricultural management influences the root microbiome. Seghers *et al.,* [25] appear to be one of the first to examine effects of agricultural practices on root microbial communities, and they reported only communities of maize root endophytic type I methanotrophic bacteria responded to different fertilizer treatments (mineral and compost), while general bacteria and fungi communities were unaffected. More recently, significant differences between lower-intensity conventional and organic farming were observed in root-associated bacteria communities in rice [26] and between different levels of N fertilizer inputs

in sugarcane [27]. Conversely, no significant differences between conventional and organic management was reported for root-associated bacteria communities in maize [28] or total root-associated fungi communities in wheat; although one specific fungal order, the Sebacinales, were shown to be nearly exclusive to organically managed root samples [29]. Thus, our results show that agricultural management affects soil and root microbial communities differently and are in accordance with a growing body of knowledge suggesting management effects on root microbial communities vary by plant species. From a management perspective, these results suggest that effects on both soil and root microbiomes must be considered when deciding which agricultural practices will be applied at a site because practices that result in changes in one microbial community compartment may not necessarily result in changes of the same magnitude in another, and the effects will vary on the crop species being cultivated. Because microbiome management is often considered with the goal of trying to promote the presence of beneficial organisms and the absence of detrimental ones, these results also stress the need to determine which specific soil and root microbiome taxa are affected by different agricultural practices and what their importance and potential function is in their respective communities for many different crop species.

The first studies documenting the effects of agriculture on soil biodiversity used lower resolution molecular fingerprinting methods which provide insights into general patterns of major microbial groups, but suffer from the inability to examine how specific microbial taxa change [30]. A benefit of using NGS technology is the improved resolution to uncover patterns in microbial diversity previously unavailable with older methods [31]. By utilizing the power of NGS and combining analytical tools like indicator species analysis and statistical testing, we detected specific shifts in the abundance of certain bacteria and fungi OTUs in response to the different cropping systems, and we termed them *c*ropping *s*ensitive OTUs (*cs*OTUs). Such methods could help to improve our ability to monitor soil and root microbiomes for the presence of beneficial and/or absence of detrimental microbes [32]. For example, we found that a number of potential fungal pathogens, based on their taxonomy assignment, had a higher abundance in soil and root samples from reduced and no-tillage practices. Although one interpretation of this finding could be that more intensive tillage of soil can reduce the abundance of pathogenic microbes, such an interpretation is too simplistic because, for example, more intensive tilling of soils has been shown to reduce diversity of beneficial fungi like AMF [33,34] and other general soil fungi [35]. Moreover, a microbe's function cannot always be reliably predicted based solely on its taxonomy assignment [36], and pathogens can remain dormant in the rhizosphere, having no apparent detrimental effects, until activated by

molecular signals from the roots of a host plant [37,38]. Thus, this serves as an example of how decisions to manage microbiomes based solely on taxonomy-based community surveys must be carefully weighed with the potential effects such actions could have on other organisms.

Co-occurrence networks identify keystone microbiome members

The above recommendation is of particular importance because members of a microbiome not only interact with their plant host or surrounding soil environment but also with each other. From the perspective of microbiome management, it is worthwhile to also know how the various members of a microbial community associate with each other and begin to unravel which community members respond to management practices and their importance in a community context. Identifying these potentially important players and developing ways to explore their function is a key step in defining which microbial functions can be manipulated through microbiome management. To begin to uncover how the different members of the soil and root microbiome associate with each other and whether these associations are influenced by the different cropping practices, we conducted co-occurrence network analyses. In both soil and root networks, we found that *cs*OTUs responding similarly to the different cropping systems were located in distinct modules of co-occurring OTUs. This was in accordance with our finding from the β-diversity analyses that found the different cropping systems harbored distinct soil and root microbiomes. This shows that agricultural management of soils not only influences the community structure of the soil and root bacteria and fungi communities, but also shapes their co-occurrence patterns.

Microbiome members in a network that frequently co-occur with many others may be considered keystone taxa and play an ecologically important role in the microbiome by determining community structure and dynamics [39,40]. As in the β-diversity analysis, where we noted that soil and root microbiomes responded differently to the cropping systems, we also found that the potential keystoneness of the *cs*OTUs differed between the soil and root communities. In the soil network, the *cs*OTUs exhibited a low to medium connectivity (low node degree), and were not identified as keystone OTUs, suggesting that despite being a significant driver of community composition, the different cropping systems largely influence less influential members of the soil microbiome. Conversely, in the root microbiome, we found that five keystone OTUs were also members of the bacteria *cs*OTUs and were more abundant in organically managed plots. Three of these OTUs were classified as *Peptostreptococcaceae* and *Erysipelotrichaceae* in the Firmicutes, a bacteria phylum common in organically managed fields in Switzerland [32] and have been isolated from the waste of livestock and other animals

[41–43]. This is particularly interesting because cattle slurry serves as fertilizer in organic plots of the FAST site [44], and thus this finding suggests that slurry application may introduce consortia of bacteria playing a keystone role into the winter wheat root microbiome. However, this should be investigated further by, for example, including samples of animal slurry in sequencing runs and identifying slurry derived OTUs in the root microbiome based upon comparisons of sequence similarity.

It is important to note that conclusions drawn from network analyses on microbial relationships are dependent on methods utilized to build the co-occurrence networks [45,46]. Thus, the results and conclusions presented here are somewhat limited by our chosen approach. For example, the positive co-occurrence relationships presented here only represent pairwise mathematical correlations between the ranked relative abundances of microbial taxa. These relationships can result from true ecological interactions between microbes which include mutualism and commensalism, but also indirect associations like niche overlap [47,48]. To develop a more holistic view of interactions in the microbiome, future studies could also include negative co-occurrence patterns in network analyses. Including such relationships increases the complexity of the resulting network but could reveal direct interactions like predation-parasitism, amensalism, or more indirection associations through resource competition, preference for mutually exclusive environmental conditions or negative allelopathy [49]. Furthermore, our use of ranked correlations only considers linear associations between microbes. Alternative statistical methods, like the maximal information criterion, can be used to reveal the strength of both linear and non-linear associations between microbial relative abundances [50] and would shed further light on cropping system effects on co-occurrence patterns or reveal new, novel microbial associations.

Considerations for future studies

There are also additional insights to be gained from further study of the FAST site. For instance, few AMF reads were found in the soil and root fungi community profiles, despite the fact AMF typically make up a large proportion of microbial biomass in soils [51] and form associations with 80% of terrestrial plant families, including winter wheat [52]. This is likely the result of our use of general ITS primers which have been shown to result in a low recovery of AMF sequences and poorly discriminate between AMF taxa [53,54]. Given that AMF have been shown to be important plant P and N acquisition [55], reduce nutrient leaching [56], reduce N_2O emissions from soil [57], and generally contribute to ecosystem productivity and stability [58,59] their role in agroecosystem functioning means their diversity patterns should

be further explored at the FAST site using AMF specific primers [54]. Additionally, future studies could take advantage of the FAST experiment's replicated design and cover crop treatments to gain more statistical power to examine differences at the landscape scale and explore the influence of cover crops on soil and root microbial communities. The effect of cover crops on soil and root microbial communities might be particularly interesting given that Wittwer *et al.,* [44] showed that the use of cover crops increased crop yield in organically managed plots at the site. While this yield increase was attributed to N-fixing cover crops, the possible microbial contributions to this finding (e.g. via rhizobia or other microbes that contribute to N cycling) certainly merits further investigation.

Perhaps the most promising avenue of future research at the FAST site is elucidating the possible function of the identified *cs*OTUs and keystone microbiome members. This represents a high research priority because of the potential such microbes have to contribute to plant and ecosystem functioning and shaping the microbiome. For example, Banerjee *et al.,* [60] used co-occurrence network analysis of bacteria and fungi communities in agricultural soils to identify keystone taxa and showed their abundance played a key role in mediating organic matter decomposition. Agler *et al.,* [39] identified two keystone microbes- an oomycete pathogen and a yeast fungus- in the phyllosphere (leaf-associated) microbiome of *Arabadopsis thaliana*. They demonstrated that these likely keystone species are major determinants of phyllosphere microbiome structure by acting as mediators between the plant and the rest of its microbiome, and effects of plant-host genotype or other abiotic factors on these keystone microbes are then cascaded throughout the rest of the microbiome. In light of these findings, experiments in which the presence or abundance of identified *css*OTUs and/or keystone taxa in the soil or root microbiome are manipulated and the effects on plant health, soil fertility, and microbiome structure of agriculturally relevant crop species are scored is a logical next step in improving our understanding of how we can manage the microbiome to improve agricultural production. These investigations will require reference stocks of keystone and *css*OTUs isolates for inoculation experiments and model systems in which such experiments can be conducted. The overall impact of such experiments could be further strengthened by integrating various "meta-omics" sequencing approaches [61]. For example, meta-transcriptomic sequencing of environmental soil or root samples could not only reveal differences in the metabolically active microbial community between the different cropping systems, but also shed light on the metabolic requirements of different microbiome members and allow for the development of culture media to target previously unculturable microbes [61,62]. Moreover, meta-transcriptomic sequencing of the inoculated microbial community in microbiota

manipulation experiments can help to shed light on the mechanisms through which certain microbiome members contribute to plant growth or other ecosystem processes (i.e. active transcription of genes encoding for certain secretion systems or nutrient solubilization). While the use of "meta-omics" approaches were beyond the scope of the work presented in this thesis, these tools could certainly complement the reference stock and microcosm systems presented in Chapters 3 and 4.

Towards experimental manipulation of the root microbiome

The development of model systems to investigate plant-microbiome interactions has been proposed as a major research priority for capitalizing on the power of the microbiome to improve agricultural sustainability [13]. These model systems are valuable for exploring the factors affecting microbiome assembly as well as microbial function in a community context [63]. Chapter 3, explored this by utilizing culture independent and dependent methods to characterize the root microbiome of *Trifolium pratenese* (red clover) and presented a reductionist microcosm system to begin to test root microbiome assembly and the function of specific root microbiome members. Although not a food crop, Trifolium still has wide agricultural application due to its use as a forage crop [64] and its classification as a legume, which are included in grass-clover mixtures to increase the N content of the soil [65]. Indeed, the results of the culture independent NGS profiling of root bacteria communities provided an estimate of ~70% of Trifolium's root microbiome comprises N-fixing rhizobia bacteria. We also detected bacteria from the genera *Pantoea, Sphingomonas, Novosphingobium,* and *Pelomonas,* whose relative abundances were significantly higher in root samples compared to bulk soil. Previous experiments with isolates of bacteria from these genera were previously shown to have plant growth promotion [66], nutrient solubilization [67], and pathogen antagonism abilities [68,69]. Taken together, our findings based on taxonomic surveys of the Trifolium root microbiome are in accordance with reports that members of the root microbiome can provide a wide variety of services to the host plant [70], and these abundant taxa provide target candidates for culturing and functional testing in future experiments. However, one must be cautious with such an interpretation because, as previously mentioned, a taxonomic assignment does not always confer microbial function, and the role of a microbe in the microbiome depends on a number of biotic and abiotic factors and microbial and host plant genotype [71].

Reductionist microcosm systems to explore root microbiome assembly and function

One way of elucidating microbial function at an individual level or in a community context is building collections of isolates and subsequently conducting inoculation experiments [61,63,72]. With this in mind, we isolated bacteria to estimate the culturable fraction of the root microbiome and to build a reference stock of bacteria isolates for functional experiments. In total, our reference stock contained 200 isolates, and based upon comparisons of sequence similarity between isolate DNA sequences and OTUs from the root community profiles, we concluded that ~24% of the most abundant root microbiome members are culturable. Although it is estimated that 99% of soil microbes are unculturable [73], our findings complement a number of recent isolation efforts reporting higher isolation rates from both soil and roots. For example, VanInsberghe *et al.,* [74] reported a collection of ~1200 bacteria isolates, representing approximately 20% of pyrotags in a forest soil. A collection of only 27 bacteria isolates isolated from maize roots represented ~48% phylogenetic overlap with a bacteria clone library [75]. More recently, Bai *et al.,* [76] conducted a large-scale isolation effort and amassed a collection of ~6,000 bacteria isolates from the roots of *A. thaliana* and calculated a 54-65% recovery rate of the most abundant root OTUs. We utilized simple isolation techniques and one culture medium to quickly build a reference stock for use in inoculation experiments. Thus, the size and diversity of the bacteria reference stock and estimates of the culturable fraction of the root microbiome could certainly be improved by further isolation efforts utilizing different culture media.

We developed a microcosm system in combination with an artificial growth substrate for first tests of specific members of the Trifolium root microbiome. However, because edaphic factors, growth conditions, and the starting microbial community can have a marked influence on the composition of the root microbiome, we also recognized the need to define the root microbiome of Trifolium growing in an artificial substrate in order to choose rational microbiome members for the functional tests. We found that the root microbiome of microcosm-grown Trifolium is markedly different than that of plants grown in native soil, despite being exposed to largely the same start community, which we inoculated via a specially prepared soil microbial extract. These results further stress the importance of edaphic factors in selecting for root microbiome composition and present new challenges when considering the transferability of results gained from microcosm experiments to the greenhouse or field. However, such experiments are still important because characterizing the microbiomes of plants grown under a variety of conditions and in different soil types can help to identify microbial taxa that are common across changing environments. Such microbes may be

members of a plant's "core" microbiome [77] and are prime candidates for focused culturing efforts and functional tests because of the services they might provide to their associated host [63].

Although characterizing and testing the function of the core microbiome of Trifolium was beyond the scope of Chapter 3, we rationalized our choice of microbial strains for functional testing in the microcosms based on bacteria that were culturable members of the abundant community in roots grown in native soil and in the microcosms. We inoculated a *Flavobacterium*, *Janthinobacterium*, *Pseudomonas,* and because it was abundant in the culture collection and common in root microbiomes of other plant species [77], a *Microbacterium* strain in monocultures and in a combined mini community in three replicate experiments. Although much research has focused on the discovery of plant growth promoting microbes [78,79], our results revealed the presence of a potential pathogen, the *Flavobacterium*, which reduced the biomass of Trifolium when grown in a monoculture, even though the plants showed no visible signs of infection (e.g. discoloration of leaves). Thus, our results reiterate the fact that not all root microbiome members have a positive effect on their host plant. However, when the *Flavobacterium* was inoculated with other community members, we found the negative growth effect was alleviated. Because our experiment was not designed to test the mechanism behind this finding, it was unclear if the *Flavobacterium* strain was actively counteracted by one or more of the other inoculated isolates though a variety of known bio-control mechanisms [80]. Further experiments and molecular characterization, through the use of "meta-omics" sequencing approaches, of the reference stock isolates will be required to determine if the observed pathogenicity of the *Flavobacterium* is strain specific or generally common of the genus and to screen for candidate strain(s) that may possess antagonistic traits (e.g. genes for antibiotic production or interference of virulence factors). This information could be useful for the discovery and development of new biocontrol strains. Moreover, the diversity of the reference stock could be further utilized to explore the relationship between root microbiome diversity and plant health or patterns of root microbiome assembly while manipulating variables like host plant genotype, resource availability, or growth conditions. Experiments to uncouple the effects of these variables on root microbiome function will lead to a greater understanding of how the root microbiome can be successfully manipulated under dynamic field conditions for agricultural benefits.

A holistic approach to explore microbial contributions to ecosystem multifunctionality

Chapter 3 used a combination of culture independent and dependent methods to characterize the composition of the Trifolium root microbiome and reductionist microcosms to investigate the contributions of specific, culturable root microbiome members to plant growth. However, such an approach is not without its shortcomings. For example, the small size of the microcosms makes performing functional experiments with larger crop species, like wheat or maize, challenging, and their current design limits the types of ecosystem functioning data that can be collected. Given that microbes are known to contribute to a wide variety of important ecosystem processes, it is worthwhile to employ a more holistic approach to investigate how altering microbiome diversity also affects other ecosystem functions. Moreover, the more homogenous growth conditions provided by the nutrient solution and artificial soil substrate in the reductionist microcosms somewhat constrain the real-world applicability of the results. Thus, developing and testing experimental systems that mimic the heterogeneity of field conditions is a logical next step in investigating the importance of the microbiome. Chapter 4 began to address this need by using larger microcosms filled with a mixture of sand and field soil and inoculated with a more complex microbial community. In this experiment, we aimed to manipulate soil bacteria and fungi communities and investigated the consequences on four individual ecosystem functions and overall ecosystem multifunctionality.

Most terrestrial ecosystems are dominated by bacteria, fungi, or a combination of both [81] and we tried to replicate these different microbial communities in autoclaved soil by combining bacteria from the reference stock presented in Chapter 2 and fungi from a reference stock of ~200 isolates from a separate isolation effort. Although both the bacteria and fungi reference stock collections were isolated from Trifolium, we chose to grow *Lolium multiflorum* (Italian ryegrass), a common grass species in European grasslands, in the microcosms. Although Lolium is known to form an association with AMF, studies have shown that grasses are generally unresponsive to the mycorrhizal symbiosis [58,82]. Moreover, as a non-legume, Lolium does not associate with nodulating rhizobia bacteria [83]. Thus the plant is a rational choice in an experiment where both of these microbial groups would not be present in the treatments. Hence, ecosystem contributions of microbiome members beyond AMF and-rhizobia can be investigated.

Previous studies have employed a variety of techniques to achieve different levels of microbial diversity in microcosms to investigate effects on ecosystem functioning. These include soil fumigation [84], inoculation of diluted soil suspensions [85,86], or sieving soil through progressively smaller sieves to create a soil biodiversity gradient [87,88]. One major

goal of the microcosm experiment was to test whether we could alter the bacteria and fungi richness and community composition of the experimental treatments through inoculation of bacteria and fungi isolates into autoclaved soil. We found no differences in inoculated bacteria richness between the different microbial treatments, with the exception of the positive control, which received unsterilized field soil. We were more successful in inoculating fungi strains into the microcosms, as our results showed higher inoculated fungi OTU richness in microbial treatments receiving the fungal inoculum, and this result provides a starting point for further experimentation that could focus on inoculating different fungal communities into microcosms and investigating the consequences for litter decomposition or nutrient cycling and retention.

Despite the apparent success with the inoculated fungi community, we still recognize that inoculated OTUs were detected in microcosms not receiving inoculum, and the total bacteria and fungi OTU richness was only significantly different in the positive control treatment. This was in spite of repeated autoclaving and careful efforts to prevent outside contamination of the experiment. These extraneous OTUs could be a result of outside or unintentionally inoculated contamination, DNA from dead or non-active organisms [89], or spurious artefacts from the sequencing and/or bioinformatics (e.g. OTU clustering) steps [90]. It is also worth noting that we also observed extraneous OTUs present in the microcosm experiment presented in Chapter 2, and thus this presents a major obstacle to overcome in future microcosm studies. However, given the ubiquitous nature of microbial life, some level of contamination may have to be accepted and its impact on richness and diversity estimates minimized through advances in sequencing technology, bioinformatics tools, and statistical analysis methods.

Another major goal of Chapter 4 was to explore if altering bacteria and fungi richness and community composition influenced ecosystem functioning. Despite some difficulty with the bacteria and fungi inoculation, as discussed above, we still observed differences in the bacteria and fungi community compositions in microcosms, and the design of the microcosms permitted us to collect data on multiple ecosystem functions, namely plant productivity, litter decomposition, leaching volume, and N-N_2O losses. The observation that decomposition was highest in the positive control treatment, where both bacteria and fungi richness were highest, nicely complements previous findings in similar microcosm studies suggesting that a greater diversity of soil organisms can enhance litter breakdown [87,88]. A logical next step would be to begin to explore the possible contributions of specific bacteria or fungi taxa to decomposition processes by, for example, NGS of DNA extracted from litter samples and testing for enriched OTUs, which could reveal novel insights into the relationships between the abundances of

specific taxa and higher rates of decomposition. Moreover, when combined with reference stocks of bacteria and fungi isolates, hypotheses developed from analysis of NGS data could be empirically tested in the reductionist microcosm systems presented in Chapter 2.

When we combined all the individual ecosystem functions into one multifunctionality index, we found lower overall ecosystem in the mixed bacteria/fungi treatment compared to the control and positive control treatments. However, this finding must be cautiously interpreted because we speculate this could be the result of particularly large $N\text{-}N_2O$ losses in some microcosms. This highlights one of the drawbacks of the averaging method we used to calculate ecosystem multifunctionality, namely it gives all individual ecosystem functions equal weighting, and thus some particularly responsive individual functions may have a large impact overall [91]. Recent work has demonstrated that effects of soil biodiversity on ecosystem multifunctionality can depend on the methodology used [88]. Moreover, giving equal weight to each ecosystem function presupposes that all functions are of equal importance. From an applied management perspective, some ecosystem functions may be more desired than others. For example, farmers looking to maximize crop yield may value biomass production, or those cultivating nutrient poor soils may value decomposition or reduced nutrient losses as a way to promote better internal nutrient retention. With this in mind, future studies would greatly benefit from assessing more individual ecosystem functions (e.g., nutrient leaching and turnover) and explore alternative methods of calculating multifunctionality indices [91] in order to provide more meaningful results about the relationship between how changing bacteria and fungi communities affect ecosystem multifunctionality.

Conclusion

Soil and plant root microbiomes harbor great potential to improve plant productivity and increase the sustainability of agricultural production. However, translating this potential into tangible benefits requires an in-depth understanding of the functions of microbiome members, both individually and in a community context, and how the microbiome can be managed to promote these functions. This thesis has demonstrated that agricultural management can shift the community composition of both the soil and root microbiome, and specific agricultural practices may permit the manipulation of highly influential microbes that play an important role in determining microbiome structure. An important next step is determining what microbial functions can be targeted through different agricultural practices. With the isolation of reference stocks of bacteria and fungi and the development of two different microcosms systems, this thesis outlines a promising approach for this future work in

which microbiome members from any plant species or soil can be isolated and the function of specific microbiome members can be tested in isolation or the diversity of the microbiome manipulated for a more holistic assessment of its importance on overall agroecosystem functioning. Although beyond the scope of this thesis, various meta-genomic sequencing approaches could also be utilized to further complement the experimental approaches presented here. Only with such systematic investigations can we begin to unravel the complex interactions between plants, the environment, and their microbiomes and provide implementable solutions for improving the future of agricultural production.

References

1. Pingali P. Green Revolution:Impacts, Limits, and the path ahead. Proc. Natl. Acad. Sci. 2012;109:12302–8.

2. Hunter MC, Smith RG, Schipanski ME, Atwood LW, Mortensen DA. Agriculture in 2050: Recalibrating targets for sustainable intensification. Bioscience. 2017;67:386–91.

3. Thomson K. World agriculture: towards 2015/2030: an FAO perspective. Land use policy. Rome; 2003. Report No.: 12.

4. Tilman D. Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices. Proc. Natl. Acad. Sci. U. S. A. 1999;96:5995–6000.

5. Cordell D, Drangert JO, White S. The story of phosphorus: Global food security and food for thought. Glob. Environ. Chang. 2009;19:292–305.

6. Cordell D, White S. Peak phosphorus: Clarifying the key issues of a vigorous debate about long-term phosphorus security. Sustainability. 2011;3:2027–49.

7. Snyder CS, Bruulsema TW, Jensen TL, Fixen PE. Review of greenhouse gas emissions from crop production systems and fertilizer management effects. Agric. Ecosyst. Environ. 2009;133:247–66.

8. Matson P, Parton W, Power A, Swift M. Agricultural Intensification and Ecosystem Properties. Science. 1997;277:504–9.

9. Pimentel D, Harvey C, Resosudarmo P, Sinclair K, Kurz D, McNair M, et al. Environmental and economic costs of soil erosion and conservation benefits. Science. 1995;267:1117–23.

10. Vitousek PM, Mooney HA, Lubchenco J, Melillo JM. Human Domination of Earth' s Ecosystems. Science. 1997;277:494–9.

11. Stoate C, Boatman ND, Borralho RJ, Carvalho CR, de Snoo GR, Eden P. Ecological impacts of arable intensification in Europe. J. Environ. Manage. 2001;63:337–65.

12. Bommarco R, Kleijn D, Potts SG. Ecological intensification: Harnessing ecosystem services for food security. Trends Ecol. Evol. 2013;28:230–8.

13. Busby PE, Soman C, Wagner MR, Friesen ML, Kremer J, Bennett A, et al. Research priorities for harnessing plant microbiomes in sustainable agriculture. PLOS Biol. 2017;15:e2001793.

14. Bender SF, Wagg C, van der Heijden MGA. An Underground Revolution: Biodiversity and Soil Ecological Engineering for Agricultural Sustainability. Trends Ecol. Evol. 2016;31:440–52.

15. Sugiyama A, Vivanco JM, Jayanty SS, Manter DK. Pyrosequencing Assessment of Soil Microbial Communities in Organic and Conventional Potato Farms. Plant Dis. 2010;94:1329–35.

16. Chaudhry V, Rehman A, Mishra A, Chauhan PS, Nautiyal CS. Changes in Bacterial Community Structure of Agricultural Land Due to Long-Term Organic and Chemical Amendments. Microb. Ecol. 2012;64:450–60.

17. Schneider S, Hartmann M, Enkerli J, Widmer F. Fungal community structure in soils of conventional and organic farming systems. Fungal Ecol. 2010;3:215–24.

18. Upchurch R, Chiu CY, Everett K, Dyszynski G, Coleman DC, Whitman WB. Differences in the composition and diversity of bacterial communities from agricultural and forest soils. Soil Biol. Biochem. 2008;40:1294–305.

19. Drijber RA, Doran JW, Parkhurst AM, Lyon DJ. Changes in soil microbial community structure with tillage under long-term wheat- fallow management. Soil Biol. Biochem. 2000;32:1419–1430.

20. Wang Z, Chen Q, Liu L, Wen X, Liao Y. Responses of soil fungi to 5-year conservation tillage treatments in the drylands of northern China. Appl. Soil Ecol. 2016;101:132–40.

21. Francioli D, Schulz E, Lentendu G, Wubet T, Buscot F, Reitz T. Mineral vs. organic amendments: Microbial community structure, activity and abundance of agriculturally relevant microbes are driven by long-term fertilization strategies. Front. Microbiol. 2016;7:10.3389/fmicb.2016.01446.

22. Zhen Z, Liu H, Wang N, Guo L, Meng J, Ding N, et al. Effects of manure compost application on soil microbial community diversity and soil microenvironments in a temperate cropland in China. PLoS One. 2014;9:e108555.

23. Lentendu G, Wubet T, Chatzinotas A, Wilhelm C, Buscot F, Schlegel M. Effects of long-term differential fertilization on eukaryotic microbial communities in an arable soil: A multiple barcoding approach. Mol. Ecol. 2014;23:3341–55.

24. Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and plant health. Trends Plant Sci. 2012;17:478–86.

25. Seghers D, Wittebolle L, Top EM, Verstraete W, Siciliano S. Impact of agricultural practices on the Zea mays L. endophytic community. Appl. Environ. Microbiol. 2004;70:1475–82.

26. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, et al. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc. Natl. Acad. Sci. 2015;112:E911–20.

27. Yeoh YK, Paungfoo-Lonhienne C, Dennis PG, Robinson N, Ragan MA, Schmidt S, et al. The core root microbiome of sugarcanes cultivated under varying nitrogen fertiliser application. Environ. Microbiol. 2015;18:1338–51.

28. Peiffer JA, Spor A, Koren O, Jin Z, Tringe SG, Dangl JL, et al. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. U. S. A. 2013;110:6548–53.

29. Verbruggen E, Rillig MC, Wehner J, Hegglin D, Wittwer R, van der Heijden MGA. Sebacinales, but not total root associated fungal communities, are affected by land-use intensity. New Phytol. 2014;203:1036–40.

30. Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G. Microbial diversity and soil functions. Eur. J. Soil Sci. 2003;54:655–70.

31. Navas-Molina JA, Peralta-Sánchez JM, González A, McMurdie PJ, Vázquez-Baeza Y, Xu Z, et al. Advancing our understanding of the human microbiome using QIIME. Methods Enzymol. 2013; 531:371-444

32. Hartmann M, Frey B, Mayer J, Mäder P, Widmer F. Distinct soil microbial diversity under long-term organic and conventional farming. ISME J. 2014;9:1177–1194.

33. Jansa J, Mozafar A, Anken T, Ruh R, Sanders IR, Frossard E. Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza. 2002;12:225–34.

34. Säle V, Aguilera P, Laczko E, Mäder P, Berner A, Zihlmann U, et al. Impact of conservation tillage and organic farming on the diversity of arbuscular mycorrhizal fungi. Soil Biol. Biochem. 2015;84:38–52.

35. Simmons BL, Coleman DC. Microbial community response to transition from conventional to conservation tillage in cotton fields. Appl. Soil Ecol. 2008;40:518–28.

36. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 2013;31:814–21.

37. Mendes R, Garbeva P, Raaijmakers JM. The rhizosphere microbiome: Significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol. Rev. 2013;37:634–63.

38. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. the Role of Root Exudates in Rhizosphere Interactions With Plants and Other Organisms. Annu. Rev. Plant Biol. 2006;57:233–66.

39. Agler MT, Ruhe J, Kroll S, Morhenn C, Kim ST, Weigel D, et al. Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. PLoS Biol. 2016;14:e1002352.

40. van der Heijden MGA, Hartmann M. Networking in the Plant Microbiome. PLOS Biol. 2016;14:e1002378.

41. Ziemer CJ. Newly cultured bacteria with broad diversity isolated from eight-week continuous culture enrichments of cow feces on complex polysaccharides. Appl. Environ. Microbiol. 2014;80:574–85.

42. Shanks OC, Kelty CA, Archibeque S, Jenkins M, Newton RJ, McLellan SL, et al. Community structures of fecal bacteria in cattle from different animal feeding operations. Appl. Environ. Microbiol. 2011;77:2992–3001.

43. Verbarg S, Göker M, Scheuner C, Schumann P, Stackebrandt E. The Families Erysipelotrichaceae emend., Coprobacillaceae fam. Nov., and Turicibacteraceae fam. Nov. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes: Firmicutes and Tenericutes. 4th ed. Berlin-Heidelberg: Springer-Verlag; 2014. p. 79–105.

44. Wittwer RA, Dorn B, Jossi W, van der Heijden MGA. Cover crops support ecological intensification of arable cropping systems. Sci. Rep. 2017;7:10.1038/srep41911.

45. Williams RJ, Howe A, Hofmockel KS. Demonstrating microbial co-occurrence pattern analyses within and between ecosystems. Front. Microbiol. 2014;5:1–10.

46. Weiss S, Van Treuren W, Lozupone C, Faust K, Friedman J, Deng Y, et al. Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. ISME J. 2016;1–13.

47. Faust K, Raes J. Microbial interactions: from networks to models. Nat. Rev. Microbiol. 2012;10:538–50.

48. Fuhrman JA. Microbial community structure and its functional implications. Nature. 2009;459:193–9.

49. Fuhrman JA, Cram JA, Needham DM. Marine microbial community dynamics and their ecological interpretation. Nat. Rev. Microbiol. 2015;13:133–46.

50. Reshef DN, Reshef YA, Finucane HK, Grossman SR, Mcvean G, Turnbaugh PJ, et al. Detecting Novel Associations in Large Data Sets. Science. 2011;334:1518–24.

51. Olsson PA, Thingstrup I, Jakobsen I, Bååth E. Estimation of the biomass of arbuscular mycorrhizal fungi in a linseed field. Soil Biol. Biochem. 1999;31:1879–87.

52. Smith SE, Read DJ. Mycorrhizal Symbiosis. 2nd ed. Academic Press; 1997.

53. Schlaeppi K, Bender SF, Mascher F, Russo G, Patrignani A, Camenzind T, et al. High-resolution community profiling of arbuscular mycorrhizal fungi. New Phytol. 2016;212:780–91.

54. Stockinger H, Krüger M, Schüßler A. DNA barcoding of arbuscular mycorrhizal fungi. New Phytol. 2010;187:164.

55. van der Heijden MGA, Martin FM, Selosse M-AA, Sanders IR. Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytol. 2015;205:1406–23.

56. Cavagnaro TR, Bender SF, Asghari HR, van der Heijden MGA. The role of arbuscular mycorrhizas in reducing soil nutrient loss. Trends Plant Sci. 2015;20:283–90.

57. Bender SF, Plantenga F, Neftel A, Jocher M, Oberholzer H-R, Köhl L, et al. Symbiotic relationships between soil fungi and plants reduce N_2O emissions from soil. ISME J. 2013;1–10.

58. van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, et al. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature. 1998;396:69–72.

59. Wagg C, Jansa J, Schmid B, van der Heijden MGA. Belowground biodiversity effects of plant symbionts support aboveground productivity. Ecol. Lett. 2011;14:1001–9.

60. Banerjee S, Kirkby CA, Schmutter D, Bissett A, Kirkegaard JA, Richardson AE. Network analysis reveals functional redundancy and keystone taxa amongst bacterial and fungal communities during organic matter decomposition in an arable soil. Soil Biol. Biochem. 2016;97:188–98.

61. Schlaeppi K, Bulgarelli D. The Plant Microbiome at Work. Mol. Plant Microbe Interact. 2015;28:212–7.

62. Bomar L, Maltz M, Colston S, Graf J. Directed culturing of microorganisms using metatranscriptomics. MBio. 2011;2:1–8.

63. Müller DB, Vogel C, Bai Y, Vorholt JA. The Plant Microbiota: Systems-Level Insights and Perspectives. Annu. Rev. Genet. 2016;50:211–34.

64. Isobe S, Kölliker R, Boller B, Riday H. Red Clover. In: Cai H, Yamada T, Kole C, editors. Genet. Genomics Breed. Forage Crop. Boca Raton, USA: CRC Press; 2014. p. 220–49.

65. Taylor NL, Quesenberry KH. Red Clover Science. Springer Science & Business Media; 1996.

66. Adhikari T, Joseph C, Yang G, Phillips D, Nelson L. Evaluation of bacteria isolated from rice for plant growth promotion and biological control of seedling disease of rice. Can. J. Microbiol. 2001;47:916–24.

67. Oliveira CA, Alves VMC, Marriel IE, Gomes EA, Scotti MR, Carneiro NP, et al. Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. Soil Biol. Biochem. 2009;41:1782–7.

68. Hahm MS, Sumayo M, Hwang YJ, Jeon SA, Park SJ, Lee JY, et al. Biological control and plant growth promoting capacity of rhizobacteria on pepper under greenhouse and field conditions. J. Microbiol. 2012;50:380–5.

69. Benítez MS, McSpadden Gardener BB. Linking sequence to function in soil bacteria: Sequence-directed isolation of novel bacteria contributing to soilborne plant disease suppression. Appl. Environ. Microbiol. 2009;75:915–24.

70. Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A. The importance of the microbiome of the plant holobiont. New Phytol. 2015;206:1196–206.

71. Hardoim PR, Overbeek LS van, Berg G, Pirttilä AM, Compant S, Campisano A, et al. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiol. Mol. Biol. Rev. 2015;79:293–320.

72. Lebeis SL, Rott M, Dangl JL, Schulze-Lefert P. Culturing a plant microbiome community at the cross-Rhodes. New Phytol. 2012;196:341–4.

73. Hugenholtz P, Pace NR. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. Trends Biotechnol. 1996;14:190–7.

74. VanInsberghe D, Hartmann M, Stewart GR, Mohn WW. Isolation of a substantial proportion of forest soil bacterial communities detected via pyrotag sequencing. Appl. Environ. Microbiol. 2013;79:2096–8.

75. Chelius MK, Triplett EW. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. 2001;41:252–63.

76. Bai Y, Müller DB, Srinivas G, Garrido-Oter R, Potthoff E, Rott M, et al. Functional overlap of the Arabidopsis leaf and root microbiota. Nature. 2015;528:364–9.

77. Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, et al. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature. 2012;488:91–5.

78. Berg G. Plant-microbe interactions promoting plant growth and health: Perspectives for controlled use of microorganisms in agriculture. Appl. Microbiol. Biotechnol. 2009;84:11–8.

79. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil. 2003;255:571–86.

80. Lugtenberg B, Kamilova F. Plant-Growth-Promoting Rhizobacteria. Annu. Rev. Microbiol. 2009;63:541–56.

81. Wardle DA, Bardgett RD, Klironomos JN, Setälä H, van der Putten WH, Wall DH. Ecological linkages between aboveground and belowground biota. Science. 2004;304:1629–33.

82. Wagg C, Jansa J, Stadler M, Schmid B, van der Heijden MGA. Mycorrhizal fungal identity and diversity relaxes plant-plant competition. Ecology. 2011;92:1303–13.

83. Gage DJ. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol. Mol. Biol. Rev. 2004;68:280–300.

84. Griffiths BS, Ritz K, Bardgett RD, Cook R, Christensen S, Ekelund F, et al. Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions : an examination of the biodiversity – ecosystem function relationship. Oikos. 2000;2:279–94.

85. Wertz S, Degrange V, Prosser JI, Poly F, Commeaux C, Freitag T, et al. Maintenance of soil functioning following erosion of microbial diversity. Environ. Microbiol. 2006;8:2162–9.

86. Wertz S, Degrange V, Prosser JI, Poly F, Commeaux C, Guillaumaud N, et al. Decline of soil microbial diversity does not influence the resistance and resilience of key soil microbial functional groups following a model disturbance. Environ. Microbiol. 2007;9:2211–9.

87. Wagg C, Bender SF, Widmer F, van der Heijden MGA. Soil biodiversity and soil community composition determine ecosystem multifunctionality. Proc. Natl. Acad. Sci. U. S. A. 2014;1–5.

88. Pellkofer S. Soil Biodiversity and Ecosystem Functioning. University of Zürich; 2015.

89. Carini P, Marsden PJ, Leff JW, Morgan EE, Strickland MS, Fierer N. Relic DNA is abundant in soil and obscures estimates of soil microbial diversity. Nat. Microbiol. 2016;2:16242.

90. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods. 2013;10:996–8.

91. Byrnes JEK, Gamfeldt L, Isbell F, Lefcheck JS, Griffin JN, Hector A, et al. Investigating the relationship between biodiversity and ecosystem multifunctionality: Challenges and solutions. Methods Ecol. Evol. 2014;5:111–24.

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PUBLICATIONS

Hartman K, van der Heijden MGA, Wittwer RA, Banerjee S, Walser, JC, Schlaeppi K. (2018). Cropping practices manipulate abundance patterns of root and soil microbiome members paving the way to smart farming. *Microbiome* 6:14. DOI: 10.1186/s40168-017-0389-9.

Ramirez K, (...), **Hartman K**, (...), et al. (2017). Detecting macroecological patterns in bacterial communities across independent studies of global soils. *Nature Microbiology*. DOI: 10.1038/s41564-017-0062-x.

Hartman K, van der Heijden MGA, Roussely-Provent V, Walser JC, Schlaeppi, K. (2017). Deciphering composition and function of the root microbiome of a legume plant. *Microbiome* 5:2. DOI: 10.1186/s40168-016-0220-z.226.

Hale BW, **Hartman, KJ**, Schoenenberger N. (2009). Condizioni e tendenze della qualità dell'acqua nel Laghetto di Muzzano, Svizzera. (Water quality status and trends in the Laghetto di Muzzano, Switzerland) *Bollettino della Società Ticinese di Scienze naturali* 97: 64-74.